FASN NEGATIVELY REGULATES NF-κB/P65 EXPRESSION IN BREAST CANCER CELLS BY DISRUPTING ITS STABILITY

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DEDICATION

I dedicate this dissertation to my wife, Christine and my Mom, Jennie, for their unending support that has made this achievement possible.

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Lincoln James Barlow

FASN NEGATIVELY REGULATES NF-κB/P65 EXPRESSION IN BREAST CANCER CELLS BY DISRUPTING ITS STABILITY

The overexpression of the multi-domain enzyme fatty acid synthase (FASN) has long been associated with poor clinical prognosis and treatment outcome in various cancers. Previous research in the Zhang lab has determined a role for FASN in mediating increases in non-homologous end-joining (NHEJ) DNA double-strand break repair activity allowing for increased cancer cell survival, and this mechanism was found to involve inhibition of NF-κB/p65. The mechanism responsible for the regulation of NF-κB/p65 by FASN in cancer cells, however, remains unknown. To this end, I was able to determine that FASN negatively regulates both the expression and activity of NF-κB/p65 in breast cancer cells, and that this effect was likely mediated by the 16-carbon saturated fatty acid palmitate, the end product of FASN catalytic activity. Specifically, FASN was found to negatively regulate p65 expression by disrupting its protein stability as a result of an increase in poly-ubiquitination of p65 protein and subsequent proteasomal degradation. Further, I found that the phosphorylation site Thr254 of p65 is involved in the regulation of p65 protein stability by FASN, in that mutation of this residue resulted in a disruption in p65 stability. Finally, I was able to determine that FASN likely inhibits the ability of the peptidyl-prolyl *cis/trans* isomerase Pin1 to assist in maintaining p65 stability, in that both siRNA

knockdown and pharmacological inhibition of Pin1 resulted in a reduction of p65 expression in FASN shRNA knockdown cells. The determination of this signaling mechanism serves to expand our understanding of the role of FASN in breast cancer cells and has the potential to assist in uncovering more effective ways to target the oncogenic FASN pathway to kill breast tumor cells and to overcome resistance to drug treatment.

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
AML	Acute myeloid leukemia
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATM	ATM serine/threonine kinase
ATP	Adenosine triphosphate
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	BRCA2, DNA repair associated protein
CBP/p300	CREB-binding protein/p300
cDNA	Complementary DNA
DAG	Diacylglycerol
DH	Beta-hydroxyacyl dehydratase
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco's modified Eagle medium
DTM	Dipentamethylene thiouram monosulfide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Enoyl reductase
ER	Estrogen receptor
FASN	Fatty acid synthase
FBS	Fetal bovine serum

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HCl	Hydrochloric acid
HDAC3	Histone deacetylase 3
HER2	Epidermal growth factor receptor
IFN-α	Interferon-alpha
IL-1	Interleukin-1
IL-8	Interleukin-8
ING4	Inhibitor of growth 4
IκB	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor
KAT8	Histone acetyltransferase 8
KR	Beta-ketoacyl reductase
KS	Beta-ketoacyl synthase
LPA	Lysophosphatidic acid
MAT	Malonyl/acetyltransferase
MEM	Minimum essential medium
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end-joining
NaCl	Sodium chloride
OA-519	Oncoantigen 519
ORF73	Human herpesvirus 8

PARP	Poly (ADP-ribose) polymerase
PARP1	Poly (ADP-ribose) polymerase 1
PBS	Phosphate-buffered saline
PDLIM2	PDZ and LIM domain protein 2
PDTC	Pyrrolidine dithiocarbamate
Pin1	Peptidyl-prolyl *cis*/*trans* isomerase NIMA-interacting 1
PIP_3	Phosphatidylinositiol-3,4,5-trisphosphate
PKA	Protein Kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PORCN	Porcupine
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
Pim-1	Proto-oncogene serine/threonine-protein kinase
Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
S1P	Sphingosine-1-phosphate
SDS	Sodium dodecyl sulfate
STAT3	Signal transducer and activator of transcription factor 3
SOCS1	Suppressor of cytokine signaling
SP1	Specificity protein 1

TBST	Tris-buffered saline and polysorbate 20
TE	Thioesterase
TNBC	Triple-negative breast cancer
TNF-α	Tumor necrosis factor-alpha
TP53	Tumor protein 53
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
Vol/vol	Volume to volume
W/V	Weight to volume
ng/mL	Nanograms per milliliter
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
USP2a	Ubiquitin-specific protease-2a
μCi/mL	Microcurie per milliliter
μg/mL	Micrograms per milliliter

Chapter 1: Introduction

1.1 Breast Cancer

1.1.1 Overview

Expected to be diagnosed in nearly 270,000 women in 2019 and account for the deaths of almost 42,000 women, the earliest documentation of breast cancer dates back to about 3500 BC as a result of the ability of breast tumors to be discovered upon physical examination (Lukong 2017). Despite the advent of modern medicine and the progression of the practice of surgical resection, as well as the development of therapies for treatment, cancer remains the second leading cause of death in women behind heart disease, wherein only lung cancer is more deadly than breast cancer.

1.1.2. Risk Factors and Prevention

A variety of potential risk factors are believed to be associated with the development of breast cancer. While men can develop breast cancer, women are exceedingly more likely to develop the disease. According to the American Cancer Society, men account for less than one percent of breast cancer diagnoses. Also, age is known to be a significant factor impacting the development of breast cancer in that fewer than five percent of women diagnosed with breast cancer in the U.S are younger than 40.

Genetic factors are also known to play a role in the development of the disease in that a subset of breast cancers is believed to be hereditary. The most common mutations correlated with breast cancer development involve the DNA repair genes breast cancer type 1 susceptibility protein (BRCA1) and BRCA2, DNA repair associated protein (BRCA2). While the impact of BRCA mutations varies depending on the study, about 70% of women having a mutation in either gene are expected to develop breast cancer at some point in their lifetime (Kuchenbaecker et al. 2017). Further, other inherited mutations, though less common, can contribute to breast cancer development including ATM serine/threonine kinase (ATM), tumor protein 53 (TP53), and phosphatase and tensin homolog (PTEN).

Other risk factors for breast cancer include race (African American and Caucasian women are more likely to develop breast cancer than other races), family history of the disease, previous breast cancer diagnosis, as well as possessing dense breast tissue. Certain lifestyle factors including excessive alcohol consumption, obesity, as well as the use of hormone therapy after menopause are also believed to contribute to a risk of developing breast cancer.

1.1.3. Development and Progression

Breast cancers arise from two main areas – the milk ducts of the breast that transport breast milk (ductal carcinoma) and the milk-producing lobules (lobular carcinoma). Despite this difference in origin, the progression of both ductal and lobular carcinoma is quite similar. Generally, the progression is

characterized by the growth and expansion of epithelial cells within the duct or lobule until the duct or lobule is consumed by the tumor cells, eventually resulting in intravasation of these cells out of the basement membrane to induce metastasis to distant organs including the bone, lymph nodes, lungs, liver, and the brain. This process is also accompanied by genetic changes, as well as increases in angiogenesis, increased proliferation of macrophages within the tumor stroma, and the proliferation of cancer-associated fibroblasts.

1.1.4. Molecular Subtypes and Treatment Options

Breast cancer, relative even to other forms of cancer, exhibits a very high level of tumor heterogeneity. To this end, there are four main types of breast cancer, each possessing qualities that directly affect its ability to be treated effectively in the clinic. The molecular characteristics and associated treatment options for these subtypes are summarized in **Table 1**. The most common molecular subtype, accounting for about 70% of all breast cancers, is Luminal A breast cancer, which is also the most treatable form of breast cancer (Blows et al. 2010). Luminal A is characterized by the expression of hormone receptors (estrogen receptor (ER) and/or progesterone receptor (PR)) and the lack of expression of the human epidermal growth factor receptor 2 (HER2) receptor. As a result of this receptor expression, it possesses the most favorable prognosis of the subtypes and can often be effectively treated with targeted therapies such as tamoxifen and aromatase inhibitors (Haque et al. 2012).

Similar to Luminal A, Luminal B breast cancer, accounting for about 12% of breast cancers, is characterized by the expression of ER and/or PR as well. However, these breast cancers possess a high percentage of Ki67 positive cells, indicating these tumor cells are rapidly proliferating, and also can be HER2 positive (Blows et al. 2010). As a result of the highly proliferative nature of these cancers, though they can also be susceptible to hormone therapy, they exhibit poorer prognosis relative to Luminal A breast cancers.

Breast cancers that do not express ER or PR but do express HER2 are termed HER2 positive breast cancers and account for about 5% of all breast cancers (Haque et al. 2012). While HER2 type breast cancers are considered more aggressive than hormone receptor positive breast cancers and are associated with overall poorer prognosis, treatment outlook for patients with this subtype has improved with the development of HER2-targeted therapies such as trastuzumab (trade name Herceptin) (Perez et al. 2014; Goldhirsch et al. 2013; Pivot et al. 2013).

Finally, the most difficult subtype of breast cancer to treat is unequivocally triple-negative breast cancer (TNBC), which does not express any targetable receptors and makes up about 10-15% of all breast cancers (Haque et al. 2012). These cancers are almost always highly invasive forms of cancer, are lacking in distinctive histological characteristics, and can only be treated with chemotherapeutics and radiation, regardless of stage and advancement (Bianchini et al. 2016). Due to this detrimental combination of factors, TNBCs have the poorest prognosis of all breast cancer subtypes. Standard neoadjuvant

regimen for TNBC patients involves either anthracyclines, typically doxorubicin, or taxanes, typically docetaxel or paclitaxel. While about 30-40% of patients with early-stage TNBC will achieve remission on this standard neoadjuvant regimen (Minckwitz et al. 2012), nearly all patients with metastatic TNBC will ultimately succumb to their disease regardless of treatment regimen (Bonotto et al. 2014). As a result, while certain targeted therapies such as poly (ADP-ribose) polymerase (PARP) inhibitors and immune checkpoint inhibitors have shown some promise in clinical trials, TNBC remains largely untreatable and the search for effective options for treatment of these patients is ongoing.

Molecular Subtype	Percentage of Breast Cancers	Characteristics	Treatment Options
Luminal A	70%	ER+ and/or PR+ HER2- Ki67 Low	Hormone therapies (eg, tamoxifen, aromatase inhibitors)
Luminal B	12%	ER+ and/or PR+ HER2+/- Ki67 High	Hormone therapies (eg, tamoxifen, aromatase inhibitors)
HER2-Type	5%	ER- PR- HER2+	HER2-targeted therapies (eg, Herceptin)
Triple-Negative	10-15%	ER- PR- HER2-	Neoadjuvant chemotherapy and radiation

Table 1. Breast cancer molecular subtypes and treatment options.
A summary of the four main molecular subytpes of breast cancer and their prevalence of occurrence, along with descriptions of expression characteristics and available clinical treatment options.

1.2. Fatty Acid Synthase (FASN)

1.2.1. FASN Structure

FASN is a very large, enzymatic protein consisting of seven domains, six of which exhibit catalytic activity (Chirala and Wakil 2004). In order of their orientation from the N-terminus, these seven domains are β-ketoacyl synthase (KS), malonyl/acetyltransferase (MAT), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP), and the culminating catalytic enzyme, thioesterase (TE) (Maier et al. 2008). The orientation of these seven domains is shown in **Figure 1**. A highly flexible structure exhibiting multiple conformational states allows for the protein to efficiently and effectively transport the growing fatty acid chain between domains.

Figure 1. Cartoon depiction of the orientation of domains within the FASN protein crystal structure.
Within the protein crystal structure of FASN, there exists two distinct groupings of individual domains. The N-terminal grouping consists of the KS, MAT, and DH domains, which exhibit a dimeric structure, while the C-terminal grouping contains the ER, KR, ACP, and TE domains. The residues between these two domain groupings are termed the core region of the enzyme. These residues, while not enzymatic in nature, do exhibit secondary structure. Adapted from Maier et al. 2008.

1.2.2. FASN Biosynthesis Pathway

The primary function of FASN is to utilize acetyl-CoA and malonyl-CoA to generate the 16-carbon saturated fatty acid palmitate as its enzymatic product (**Figure 2**). The initial step in this process involves the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). Following this reaction, the growing fatty acid chain is elongated in seven sequential condensation reactions that add 2 additional carbons per reaction, wherein the nascent fatty acid is shuttled between enzymatic domains by ACP. This series of reactions culminates in the hydrolysis of the thioester linkage between the fatty acid chain and ACP by the TE domain and the release of the mature palmitate. The mature fatty acid is then transported throughout the cell where it is incorporated into a variety of higher order lipids which participate in a multitude of different cellular functions.

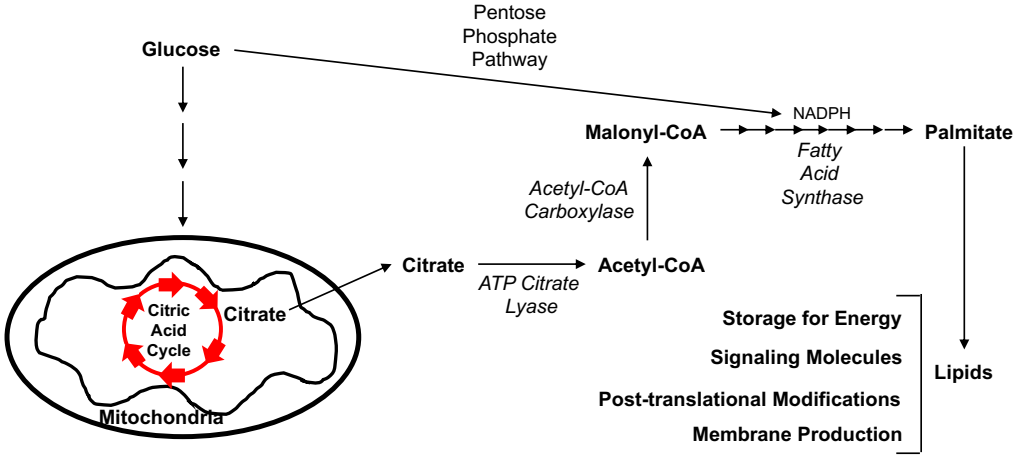


Figure 2. Mechanism of the fatty acid biosynthesis pathway. When the citric acid cycle intermediate citrate is in excess, it is shunted out of the cycle and is released from the mitochondria into the cytoplasm. Citrate is then converted to acetyl-CoA. Next, acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase. Malonyl-CoA, a two-carbon molecule, is then used in a series of seven consecutive condensation reactions, catalyzed by FASN and using NADPH as a reducing agent, which is supplied through the pentose phosphate pathway. From these reactions, the 16-carbon saturated fatty acid palmitate is synthesized and released in its mature form when it is hydrolyzed from the thioesterase domain. Palmitate is then incorporated into complex lipids including lipid signaling molecules, lipids used for energy production to meet cellular requirements via the fatty acid oxidation pathway, lipids used to post-translationally modify proteins, and for the membrane requirements of the cell. Adapted from Kuhajda 2000.

1.2.3. FASN and Cancer

In normal cells, the preferred pathway for energy production is aerobic respiration in the mitochondria, an efficient, high-yield process resulting in the production of 36 ATP. However, unlike normal cells, cancerous cells consume large quantities of glucose and preferentially utilize lactic acid fermentation for

energy production, despite their substantial energy requirements as rapidly dividing cells. Uncovered by Otto Warburg, this paradoxical phenomenon is termed the Warburg effect (Warburg 1956). The goal of this process is to rapidly generate metabolic intermediates instead of ATP in order for these intermediates to be used to satisfy the cellular requirements of cancer cells as rapidly proliferating cells.

Following Warburg's observations, it soon became clear that glucose metabolism was not the only metabolic abnormality exhibited by tumor cells. Specifically, while normal cells obtain the fatty acids necessary for their cellular requirements from dietary uptake, tumor cells undergo significant *de novo* fatty acid synthesis. Newly synthesized fatty acids have a variety of roles in cancer cells including utilization in membrane production to satisfy the substantial membrane requirements of rapidly dividing cancer cells, the production of higher order lipids to be stored for energy production through fatty acid β-oxidation, as well as the generation and modification of cellular signaling molecules.

Though this substantial increase in *de novo* fatty acid synthesis had been uncovered, a potential specific role for FASN in promoting cancer was not clear until the discovery of oncoantigen 519 (OA-519), which was subsequently identified as FASN, as a novel prognostic marker in prostate cancer (Epstein et al. 1995; Shurbaji et al. 1996). FASN was then found to also exhibit a role as a prognostic marker in breast cancer (Alo et al. 1996; Alo et al. 1999). Shortly thereafter, it was determined that FASN inhibition with cerulenin inhibited breast cancer cell growth and promoted breast cancer cell apoptosis, thereby indicating

a direct role for FASN in promoting the progression of cancer (Pizer et al. 1996). In subsequent years, studies determined this phenomenon to be consistent across many different types of cancer both *in vitro* and in *in vivo* xenograft tumor models, indicating a broader role for the involvement of FASN in tumor growth.

In addition to the role of FASN as a prognostic marker in cancer and the ability of FASN inhibition to suppress tumor growth, a role for FASN in promoting cancer drug resistance in breast cancer cells was determined (Liu et al. 2008). Specifically, FASN was significantly upregulated in drug resistant breast cancer cells, while knockdown of FASN level or inhibition of FASN pharmacologically reversed this drug resistance phenotype (Liu et al. 2008). Further supporting the contribution of FASN to drug resistance, FASN overexpression was also found to promote resistance to gemcitabine and radiation treatment in pancreatic cancer cells (Yang et al. 2011). As a result, FASN remains a potential therapeutic target for the treatment of a variety of different forms of cancer both as a potential adjuvant therapy, as well as to overcome acquired drug resistance to chemotherapeutic treatment.

1.2.4. Lipid Signaling in Cancer

As referenced earlier, the substantial increases in *de novo* lipogenesis in cancer cells can have a profound effect on cell signaling pathways. One of the most common ways FASN can affect cell signaling is through the process of protein acylation, and one key example of protein acylation impacting cell signaling events in cancer settings lies with the Wnt protein family (Rohrig and

Schulze 2016). The Wnt proteins are modified by protein palmitoylation, characterized by the addition of a palmitoyl moiety to a cysteine residue, and this enzymatic process is mediated by an O-acyltransferase known as porcupine (PORCN) (Nile and Hannoush 2016). Palmitoylation is essential for the ability of the Wnt proteins to be secreted and subsequently bind to their target receptors on the cell membrane to activate the Wnt signaling pathway, and it has been shown that blocking the action of PORCN in this process can stop cancer cell growth in Wnt-driven cancers (Abrami et al. 2008; Komekado et al. 2007; Proffitt et al. 2013). Other proteins that are palmitoylated include the Ras small GTPases, and the palmitoylation of these proteins aids in Ras trafficking and enhances Ras signaling by promoting Ras association in signaling microdomains near the membrane (Eisenberg et al. 2013).

The increased fatty acid production in cancer cells can also lead to the increased generation of lipid molecules that participate directly in cancer signaling processes. Some of the signaling lipids that have roles in mediating cancer progression include sphingosine-1-phosphate (S1P) and lysophophatidic acid (LPA), as well as second messenger molecules, such as diacylglycerol (DAG) and phosphatidylinositiol-3,4,5-trisphosphate (PIP_3) (Rohrig and Schulze 2016). With the increases in *de novo* fatty acid synthesis that are seen in cancer cells, the consequence is an abundance of free fatty acids in the cell, which allows for the rapid remodeling and reprogramming of higher order lipids into these lipid signaling molecules, and these molecules are known to play roles in many cancer-promoting signaling pathways that contribute to increased cancer

cell survival, proliferation, and angiogenesis. Therefore, lipids contribute to cancer cell growth and survival both through the modification of proteins in the cell, as well as directly via lipid signaling molecules.

1.3. Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells (NF-κB)

1.3.1. NF-κB Signaling Pathway

The NF-κB signaling pathway is fairly complex in that it can be activated by a variety of different stimuli and has many points of regulation. However, a few major molecular players predominate in the NF-κB pathway. These molecular players include p50 and RelA/p65, two proteins that form a heterodimeric complex and constitute the DNA binding proteins responsible for the transcriptional activity of NF-κB; the nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor (IκB) proteins, most specifically IκBα, which constitute the inhibitory proteins of NF-κB within the cytosol; and the IκB kinase complex, consisting of IKKα, IKKβ, and NF-κB essential modulator (NEMO), which participates in initiating the activation of the NF-κB pathway upon response to a stimulus (Gilmore 2006).

Canonically, upon the binding of a stimulus to a receptor, adaptor proteins recruit the IKK complex to the membrane. Activated IKK then phosphorylates the IκBα inhibitory subunit, inducing its poly-ubiquitination and proteasomal degradation, leaving p65/p50 free in the cytosol and allowing it to translocate to the nucleus and activate gene transcription (**Figure 3**). Due to the post-

translational modifications within this pathway and the dependence of activation on the degradation of the inhibitory subunit, NF-κB signaling is transient in nature and also very tightly regulated.

Transcriptional activation of the NF-κB signaling pathway regulates the gene expression of over 150 target genes (Pahl 1999). These genes include 27 different cytokines and chemokines, several immune receptors, genes involved in antigen presentation, cell adhesion molecules, apoptotic regulators, growth factors, stress response genes, transcription factors, many members of the NF-κB signaling pathway itself, and many other genes (Pahl 1999). Because of the complex nature of the regulation of the NF-κB signaling pathway, specific subsets of these genes will be transcriptionally activated depending in large part on the stimulus present, as well as the cell type in which activation is occurring.

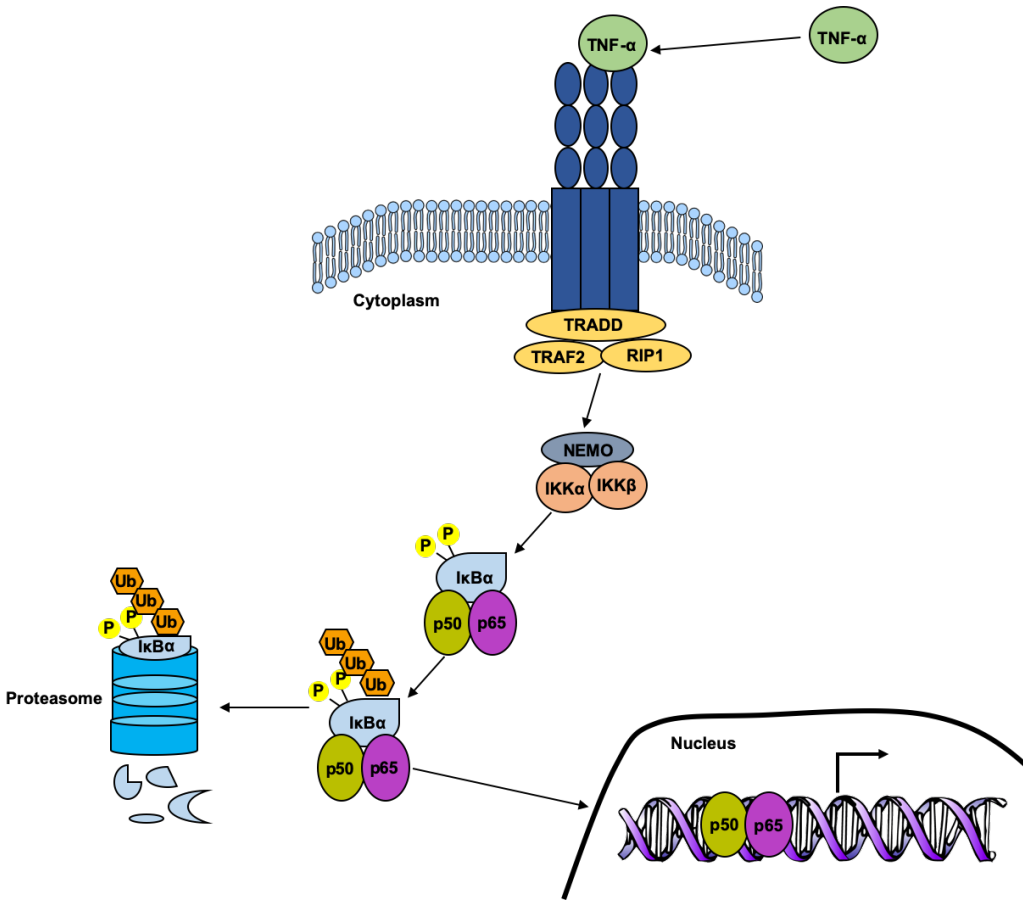


Figure 3. Canonical NF-κB signaling pathway.
Canonically, upon the binding of a stimulus to a receptor, such as the interaction between tumor necrosis factor-alpha (TNF-α) and the tumor necrosis factor receptor (TNFR), the NF-κB intracellular signaling pathway is activated, wherein adaptor proteins are recruited to the stimulated receptor at the membrane. These adaptor proteins recruit and bind the IKK complex, which is activated upon the formation of the complex. Once activated, the catalytic subunits of the IKK complex, IKKα and IKKβ, serve to phosphorylate the inhibitory subunit IκBα, which is bound to the p65/p50 heterodimer in the cytosol, at Ser32 and Ser36, leading to the poly-ubiquitination and proteasomal degradation of IκBα. The degradation of IκBα leaves the p65/p50 heterodimer free in the cytosol, thereby allowing for its nuclear translocation, wherein it binds to consensus binding elements present in target gene promoters and participates in the activation of gene transcription. Adapted from Jost and Ruland 2007.

1.3.2. NF-κB Signaling Function and Role in Cancer

The transcriptional activity of NF-κB plays an important functional role in both inflammation and cancer, and these pathological roles are also intertwined in that many genes regulated by NF-κB contribute to both pathologies. In normal conditions, the induction of NF-κB's pro-inflammatory gene expression programming results in an effective response to insult or injury within the human body that can be resolved in a timely manner (Zhang and Sun 2015; Lawrence 2009). However, dysregulation of this immune response, often involving constitutive activation of NF-κB, can lead to chronic inflammation and pathological immune-related disorders.

The relationship between NF-κB and cancer is multi-faceted. The most well-documented role of NF-κB signaling in cancer involves constitutive NF-κB activation and, specifically, the ability of NF-κB to regulate a myriad of genes that control cell proliferation, as well as apoptosis and cell survival. A long history of evidence suggests that the regulation of pro-proliferation genes and anti-apoptotic genes goes hand-in-hand to promote tumorigenesis, and there is evidence to suggest that inhibiting the activity of NF-κB can reverse the tumorigenic phenotype resulting from constitutive NF-κB activation and trigger apoptosis (Biswas et al. 2003; Karin and Lin 2002; Barkett and Gilmore 1999; Biswas et al. 2004).

The NF-κB gene expression profile responsible for tumor promotion is also believed to involve genes involved in the promotion of angiogenesis and metastasis. While the relationship between NF-κB and promotion of invasiveness

is not as well-studied as its dual role in prevention of apoptosis and promotion of proliferation, it has been shown that the NF-κB target gene interleukin-8 (IL-8) contributes to angiogenesis (Koch et al. 1992). Further, it has also been shown that inhibiting NF-κB resulted in a decrease in angiogenesis and tumor growth, as well as increased survival in mice, and was accompanied by reduced expression of both IL-8 and another angiogenic gene, vascular endothelial growth factor (VEGF) (Huang et al. 2000).

Further, aside from the somewhat general roles NF-κB signaling plays in the progression of a variety of tumor types, NF-κB signaling plays a specific role in the development of leukemias and lymphomas (Karin et al. 2002). For instance, for a specific subtype of diffuse large B-cell lymphoma (DLBCL), constitutive activation of NF-κB is, in fact, required for cancer cell viability (Bidere et al. 2009). Also, certain germline mutations in NF-κB are particularly prevalent in certain leukemias and lymphomas resulting in a unique reliance of the cancer on NF-κB or a predisposition to the development of the cancer (Leeksma et al. 2017).

In breast cancer, NF-κB has been shown to upregulate genes implicated in cancer progression, including cyclin D1, cyclin-dependent kinase 2, and c-Myc (Wang et al. 2015). NF-κB activation in breast cancer has also been specifically linked to the upregulation of cytokines, as well as anti-apoptotic and pro-survival genes. Interestingly, a link has been determined between NF-κB activation and HER2-type breast cancer, wherein HER2 overexpression leads to an upregulation of NF-κB activity resulting in increased cancer cell proliferation

(Merkhofer et al. 2010). Another study also determined that constitutive NF-κB activation led to the development of drug resistance in HER2-overexpressing breast cancer cells (Bailey et al. 2014).

It is also believed that NF-κB activation plays a particular role in breast cancer by interacting with tumor stroma and cancer stem cells to promote epithelial-mesenchymal transition and metastasis. To this end, NF-κB signaling is known to lead to the upregulation of a variety of genes in breast cancers involved in the promotion of metastasis, and this process is believed to involve the ability of pro-inflammatory signaling from the tumor microenvironment to translate to NF-κB-mediated gene expression changes (Wang et al. 2015).

Aside from the role of NF-κB in promoting drug resistance in HER2-type breast cancer, it is also believed NF-κB activation may contribute to the development of resistance to endocrine therapies such as tamoxifen, wherein crosstalk between constitutive NF-κB signaling activation and ER signaling acts to promote ER activity (Khongthong et al. 2019). Additionally, as might be expected, breast cancer cell treatment with chemotherapeutic agents including doxorubicin and taxanes such as paclitaxel has been shown to induce NF-κB activation to promote treatment resistance through the upregulation of pro-survival genes (Tergaonkar et al. 2003; Esparza-Lopez et al. 2013; Patel et al. 2000).

Interestingly, within cancer settings, NF-κB does not always participate in tumor promotion and prevention of cell death. In some cases, it has been shown that the activation of NF-κB can actually promote cell death. Specifically, it has

been shown that stimulation of NF-κB by interleukin 1 (IL-1) in co-stimulated epithelial carcinoma KB cells and keratinocytes resulted in increases in both tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Kothny-Wilkes et al. 1998), as well as UVB radiation-induced apoptosis (Strozyk et al. 2006). Also, another study showed that disruption of NF-κB signaling by either inhibiting NF-κB with pyrrolidine dithiocarbamate (PDTC) or by transfection of mutant IκBα to prevent RelA/p65 nuclear translocation resulted in decreased sensitivity of neuroblastoma cells to doxorubicin treatment, again suggesting NF-κB can play a positive role in cell death mechanisms (Bian et al. 2001). Further, transfection of an antisense IκBα vector to reduce IκBα expression, thereby inducing increased NF-κB activation, resulted in increased sensitivity of breast cancer cells to paclitaxel-induced cell death (Huang and Johnson et al. 2000). While these examples of NF-κB acting to promote cell death and apoptosis may appear isolated, there is cell signaling evidence supporting this role for NF-κB, in that DNA-damaging agents have been shown to lead to NF-κB-mediated upregulation of Fas ligand, a common activator of extrinsic apoptotic pathways, thereby suggesting further that the role of NF-κB signaling in response to chemotherapeutic treatment may be complex (Kasibhatla et al. 1998).

1.3.3. Stimulation of NF-κB by TNF-α

TNF-α is a cytokine known to play a dual role in cancer in that it can act in both a pro-survival and pro-apoptotic manner. With regard to the activation of

NF-κB, however, TNF-α is known to bind to the TNF receptor to stimulate the canonical NF-κB signaling pathway through the activation of the IKK complex. Canonical activation of NF-κB signaling by the binding of TNF-α to its receptor leads to a transcriptional response that can upregulate a variety of genes including pro-inflammatory cytokines, as well as a variety of genes involved in anti-apoptotic mechanisms (Zhou et al. 2003). The potential role for TNF-α in promoting apoptosis and cell death through NF-κB is unclear, but it has been shown that combined stimulation of colon cancer cells with TNF-α and interferon-alpha (IFN-α) resulted in increased apoptosis mediated by NF-κB-driven upregulation of Fas ligand (Kimura et al. 2003). TNF-α is not directly linked to activation of the noncanonical NF-κB pathway; however, there are several TNF family members that can regulate the noncanonical pathway, including CD40 ligand, which acts to induce activation of p52/RelB heterodimer-mediated transcription (Hayden and Ghosh 2014).

1.3.4. Regulation of NF-κB/p65

As discussed earlier, NF-κB signaling activation is very tightly regulated. While the most prominent regulatory action in the NF-κB signaling pathway involves the phosphorylation and subsequent degradation of the inhibitory subunit IκBα, NF-κB signaling can also be regulated by direct modification of the NF-κB proteins.

Specifically, phosphorylation of the NF-κB proteins has been found to play a major role in regulating the NF-κB signaling pathway. Phosphorylation of the NF-κB proteins at a variety of different sites controls transcriptional activation, protein-protein interactions, and protein stability (Christian et al. 2016). Many of these phosphorylation events occur on the NF-κB subunit p65 leading to a multitude of different effects both on p65 itself and within the cell. In fact, 11 such sites of p65 phosphorylation have been identified (Christian et al. 2016). A full list of these phosphorylation sites and their corresponding effects is displayed in **Table 2**.

Perhaps the most well-studied of these phosphorylation sites is Ser536, which is located in the transactivation domain of the protein. A number of kinases have been identified to phosphorylate Ser536, including IKKα, IKKβ, and IKKε, among others (Buss et al. 2004; Lawrence et al. 2005; Sakurai et al. 1999; Sizemore et al. 2002). One well characterized consequence of p65 Ser536 phosphorylation is the binding of the acetyltransferase CREB-binding protein/p300 (CBP/P300), resulting in increased acetylation of p65 at Lys310, and subsequently resulting in increased NF-κB transactivation (Chen et al. 2005). The effects of p65 Ser536 phosphorylation on NF-κB transcriptional activity have been described in many additional studies, and it is the p65 site most commonly associated with NF-κB activation.

Another well-characterized p65 phosphorylation site is Ser276. The main kinase involved in Ser276 phosphorylation is Protein Kinase A (PKA), and PKA phosphorylation of Ser276, similar to Ser536 phosphorylation, promotes the

interaction of p65 with CBP/P300 and results in p65 transcriptional activation (Zhong et al. 1998). Another kinase that has been shown to phosphorylate Ser276 is proto-oncogene serine/threonine-protein kinase (Pim-1) (Nihira et al. 2010). Phosphorylation of p65 at Ser276 by Pim-1 was found to not only enhance p65 transcriptional activity, but also prevent the poly-ubiquitination and proteasomal degradation of p65, thereby resulting in increased p65 protein half-life (Nihira et al. 2010).

Further, another phosphorylation site that has been found to be implicated in NF-κB activation, for which the responsible kinase is still yet unknown, is Thr254 (Xing et al. 2011; Ryo et al. 2003). Similar to the Ser276 site, when Thr254 of p65 was mutated, p65 protein stability was significantly reduced, as was the ability for p65 to translocate to the nucleus in the presence of a stimulus or activate a luciferase reporter (Ryo et al. 2003). However, mutation of a different residue also known to be involved in NF-κB activation, Ser316, did not affect p65 protein stability, indicating that Ser316 regulates NF-κB activation through a different mechanism. Further, inhibition of proteasomal degradation with the inhibitor MG132 resulted in a rescue of p65 protein following mutation of Thr254, indicating that disruption of p65 protein stability will induce its degradation through the proteasome. Immunoprecipitation studies showed that peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) was able to bind to p65, but that binding was disrupted upon Thr254 mutation, indicating that Pin1 preferentially binds to the phospho-Thr/Pro motif present at residues 254 and 255 in the p65 amino acid sequence. It was also found that loss of Pin1 resulted

in a reduction in NF-κB activity, p65 nuclear translocation, and p65 protein stability, suggesting that Pin1 is essential to p65 stability and, subsequently, NF-κB activity. Therefore, phosphorylation of many sites on the NF-κB subunit p65 tightly regulate both the transcriptional activity of NF-κB and the protein stability of p65.

Site	Kinase	Effect
S205	unknown	transactivation
T254	unknown	prolyl isomerisation, transactivation
S276	PKA-C, MSK1, MSK2, Pim-1, RSK p90, PKCα	transactivation, K310 acetylation
S281	unknown	transactivation
S311	PKCζ	K310 acetylation, transactivation
S316	unknown	transactivation
T435	unknown (CK2, PLK1?)	transactivation
S468	GSK3β	Inhibition
	IKKε	transactivation
	IKKβ	slight inhibition
T505	Chk1	inhibition
S529	CK2	transactivation
S536	IKKβ, RSK1, IKKα, IKKε, NAK/TBK1	transactivation, K310 acetylation

Table 2. Identified p65 phosphorylation sites, responsible kinases, and the functional outcomes of the phosphorylation events. Reprinted here with permission from Christian et al. 2016 under the Creative Commons Attribution License.

The termination of the NF-κB signaling response is also very tightly regulated and primarily occurs through feedback loops. These feedback loops are controlled by gene transcription and protein modification. For example, following NF-κB activation by TNF-α stimulation, the inhibitory protein IκBα is re-synthesized through NF-κB's transcriptional activity. The newly synthesized IκBα proteins then enter the nucleus and remove NF-κB proteins from their target gene promoters, resulting in the termination of gene transcription (Zabel and Baeuerle 1990). Further, proteasomal degradation can aid in the termination of the NF-κB signaling response. Ubiquitination of the p65 subunit of NF-κB can be mediated by several E3 ubiquitin ligases, including suppressor of cytokine signaling (SOCS-1) and PDZ and LIM domain (PDLIM2) (Renner and Schmitz 2009). Conjugation of ubiquitin to p65 by these E3 ligases subsequently results in the proteasomal degradation of p65 and the termination of the transcriptional response.

1.4. Peptidyl-Prolyl *Cis*/*Trans* Isomerase NIMA-Interacting 1 (Pin1)

1.4.1. Overview of Pin1 Function

Peptidyl-prolyl isomerases are a group of enzymes that affect protein folding and conformations by catalyzing the *cis*/*trans* isomerization of the peptide bonds of proline residues. As a result of the ability of the peptidyl-prolyl isomerases to affect the conformations of many proteins, their activity is linked to a variety of different cellular effects.

Pin1 is a specific isomerase that catalyzes the isomerization of proteins at phosphorylated-serine/threonine-proline motifs (Zhou et al. 1999). Because of this targeted enzymatic activity, Pin1 is known to play specific signaling roles in cells throughout the body to impact different disease states, including neurological diseases such as Alzheimer's disease, Huntington's disease, and Parkinson's disease, as well as a variety of cancers (Chen et al. 2018).

1.4.2. The Role of Pin1 in Breast Cancer

In cancers, Pin1 is often found to be overexpressed and its overexpression or overactivation is known to be correlated with a poor clinical prognosis (Driver et al. 2012; Driver et al. 2015). Specifically, with respect to breast cancer, a study found that Pin1 expression was about tenfold increased in breast tumors compared to normal tissues, and that Pin1 expression increased in proportion to increases in invasiveness of breast tumors (Wulf et al. 2001).

Pin1 is known to regulate a variety of genes and processes in breast cancer cells, including regulating proliferation both by interacting with ERα to promote ER signaling and by promoting the stability of HER2 (Rajbhandari et al. 2012; Lam et al. 2008). Pin1 is also believed to play a role in invasion and metastasis in breast cancer, in that Pin1 has been shown to increase activation of both the Notch signaling pathway and the signal transducer and activator of transcription 3 (STAT3) signaling pathway (Rustighi et al. 2009; Lufei et al. 2007). As stated earlier, Pin1 has been linked to stability of NF-κB through binding to the phosphorylated-Thr254-Pro motif of p65 and has also been linked

to other pro-survival mechanisms, including the promotion of resistance to tamoxifen treatment in ER+ breast cancer cells (Ryo et al. 2003; Namgoong et al. 2010).

1.5. Established Relationship Between FASN and NF-κB

1.5.1. FASN Regulates DNA Repair Activity via NF-κB Signaling

Concurrent with the uncovered relationship between FASN and drug resistance, it was also recently discovered that FASN overexpression results in resistance to a variety of different DNA-damaging agents, including doxorubicin, mitoxantrone, etoposide, and cisplatin, suggesting a relationship wherein FASN may desensitize cancer cells to DNA damage (Liu et al. 2013). When investigating potential signaling pathways that could be involved in this relationship, an array analysis showed that only two genes, both involved in the regulation of NF-κB, out of nearly 100 that were tested were significantly affected—TNF-α and IL-8, wherein the expression of both genes was significantly reduced with FASN overexpression in MCF7 breast cancer cells (Liu et al. 2013). This negative correlation between FASN and TNF-α expression held true both in the presence and absence of doxorubicin treatment to induce DNA damage. Further, it was also found that FASN overexpression in MCF7 cells decreased p65 protein level, while FASN knockdown, also in MCF7 cells, resulted in an increase in NF-κB activity (Liu et al. 2013). These findings indicated a potential relationship between FASN and NF-κB, wherein FASN may

prevent cancer cell death resulting from DNA damage involving inhibition of NF-κB signaling through TNF-α. This phenomenon would be consistent with the non-canonical functional outcome of NF-κB signaling described earlier, wherein NF-κB may actually promote rather than prevent tumor cell death.

Further study indicated a specific role for NF-κB in the relationship between FASN and DNA repair. FASN was found to be able to upregulate the repair of DNA double-strand breaks by increasing non-homologous end-joining (NHEJ) DNA repair activity, and this was accomplished through increasing the expression and activity of poly (ADP-ribose) polymerase 1 (PARP1) (Wu et al. 2016) (**Figure 4**). Interestingly, the expression of PARP1 was found to be regulated by a composite transcription factor binding site between specificity protein 1 (SP1) and NF-κB (Wu et al. 2016). As a result, FASN increased the expression of SP1, while decreasing the expression of NF-κB/p65 to allow for the increases in expression and activity of PARP1. Supporting the proposed relationship between NF-κB and PARP1, overexpression of p65 suppressed PARP1 expression (Wu et al. 2016). Similarly, treating cells with TNF-α to activate NF-κB also reduced PARP1 expression. Finally, knockdown of p65 resulted in an increase in NHEJ DNA repair activity, ultimately indicating that FASN inhibits NF-κB, which relieves an ability of NF-κB, through an unknown mechanism, to reduce PARP1 expression, thereby preventing the activation of the DNA repair process.

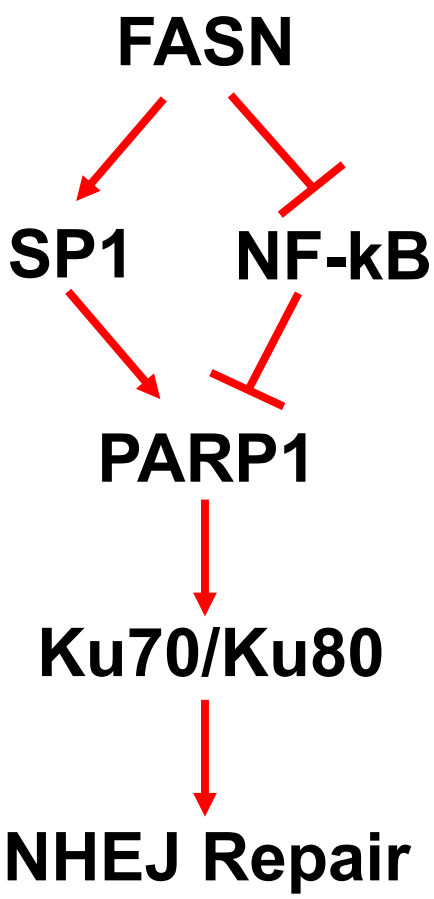


Figure 4. Mechanism of FASN regulation of NHEJ DNA repair. When FASN expression is high in breast cancer cells, FASN induces an increase in SP1 expression, while concurrently suppressing NF-κB/p65 expression. This relieves NF-κB's ability to inhibit PARP1 and allows SP1 to upregulate the expression and activity of PARP1. PARP1 then carries out its poly (ADP) ribosylation activity on various target proteins, including Ku70/Ku80 protein dimers, allowing for the retention of Ku70/Ku80 at DNA double-strand break sites. Other proteins involved in DNA repair are also recruited, and NHEJ DNA repair is carried out to repair DNA breaks and allow for the continued survival of breast cancer cells in the presence of chemotherapeutic treatment with doxorubicin and other DNA-damaging agents. Adapted from Wu et al. 2016.

Other studies have also investigated potential links between FASN and NF-κB signaling. It has been found that pharmacological inhibition of FASN results in rapid translocation of p65 to the nucleus in SK-Br-3 breast cancer cells (Menendez et al. 2004) and in both A549 and H1975 lung cancer cells (Lemmon et al. 2011). Further, FASN inhibition also led to increases in NF-κB reporter activity, as well as the expression of NF-κB target genes (Lemmon et al. 2011). In this mechanism, NF-κB activation was dependent on protein kinase C (PKC) activation, which resulted in the PKC-mediated phosphorylation of the inhibitory subunit IκBα to lead to NF-κB activation in response to FASN inhibition with C93, a derivative of another inhibitor known as cerulenin (Lemmon et al. 2011). Therefore, there exists a clear relationship between FASN signaling and NF-κB signaling within different cancer settings, both in the presence and absence of chemotherapeutic treatment and DNA damage.

1.6. Summary and Hypothesis

FASN is a metabolic enzyme that has been long characterized as having an important role in the progression of many forms of cancer, as well as being a negative indicator of disease prognosis and treatment outcomes. Previous research has uncovered a specific mechanistic role for FASN in breast cancer cells, wherein upregulation of FASN leads to increases in NHEJ DNA repair activity and, subsequently, increased breast cancer cell survival in the presence of DNA-damaging agents such as doxorubicin. While the ability of FASN to positively regulate NHEJ DNA repair activity in breast cancer cells has been

shown to involve the inhibition of NF-κB/p65, it remained unknown until now how FASN is able to regulate NF-κB to allow for FASN to exert this effect.

As a result, the goal of the study explored in this thesis was to investigate the mechanism behind FASN regulation of NF-κB/p65 in breast cancer cells. To this end, I hypothesized that FASN negatively regulates NF-κB/p65 expression in breast cancer cells by disrupting p65 protein stability. Data presented in this thesis will be shown to support that FASN negatively regulates p65 at the protein level but does not affect p65 transcription. Further, data will also demonstrate that, when FASN expression is high in breast cancer cells, p65 stability is decreased, while the inhibition of proteasomal degradation can reverse the ability of FASN to reduce p65 expression. Finally, this thesis will also show that the effect of FASN on p65 protein stability is likely due to reduced phosphorylation of p65 at Thr254 involving the activity of the prolyl isomerase Pin1.

Chapter 2: Materials and Methods

2.1. Cell Lines and Culture Conditions

Human breast cancer cell lines MDA-MB-468 and MDA-MB-231 were cultured at 37 degrees Celsius with 5% (vol/vol) CO_2 in DMEM (Thermo Fisher) supplemented with 10% (vol/vol) FBS. Drug-resistant MCF7 breast cancer cells (M3K) were generated previously (Chen et al. 1990) and were cultured in DMEM supplemented with 10% (vol/vol) FBS, 3000 ng/mL Adriamycin, and 5 μg/mL verapamil, which is used to prevent the overexpression of P-glycoprotein as a mechanism for Adriamycin resistance in this cells. The mechanisms of drug resistance that have been uncovered in this cell line since its initial selection are summarized in **Table 3**. Stable FASN-overexpressing MCF7 cells (MCF7/FASN) and its corresponding vector-transfected control cell (MCF7/Vec), as well as stable M3K cells with FASN knockdown (M3K/shFASN) and its scrambled shRNA-transfected control cell (M3K/shScr) were generated previously (Liu et al. 2008), and were maintained in DMEM supplemented with 10% (vol/vol) FBS and 400 μg/mL G418.

To generate stable clones in MDA-MB-436 cells with FASN overexpression, pcDNA(3.1) containing cDNA encoding human FASN (Liu et al. 2008) was transfected into MDA-MB-436 cells using Metafectene Pro (Biontex). Two days after transfection, cells were re-plated at a 1:10 dilution, allowed to recover for 24 hours, and selected in the presence of 600 μg/mL G418 for a period of 2 weeks. Following selection, individual clones were propagated for

continued analysis. FASN overexpression was verified by Western blot. Cultured MDA-MB-436 cells with FASN overexpression (436/FASN) were subsequently maintained in MEM, 1X (Thermo Fisher) supplemented with 10% (vol/vol) FBS and 300 μg/mL G418.

Resistance Mechanism	Resistance Category	Associated References
Unknown 95 kDa membrane protein	Drug efflux (unconfirmed)	Chen et al. 1990
BCRP/ABCG2	Drug efflux	Litman et al. 2000; Doyle et al. 1998
Other ABC Transporters	Drug Efflux	Liu et al. 2005
14-3-3σ	Cell cycle/DNA repair	Liu et al. 2006
FASN	DNA repair	Liu et al. 2008; Liu et al. 2013; Wu et al. 2016

Table 3. Summary of observed drug resistance mechanisms in M3K (MCF/AdVp3000) breast cancer cells.

2.2. Western Blotting

Cultured cells were scraped and pelleted in sterile 1X PBS via centrifugation at 5,000xg for 5 mins. Pelleted cells were then washed once in sterile 1X PBS by centrifugation at 13,000 rpm for 10 mins. Cells were subsequently frozen at -80 degrees Celsius. Upon thawing, cells were lysed in

TNN lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 0.1% SDS, 0.5 mM PMSF, 70 mM DTT] using intermittent vortexing and incubation of cells on ice. Cells were then sonicated three times for 15 seconds, followed by centrifugation at 13,000 rpm for 10 mins at 4 degrees Celsius to pellet cell debris. Supernatant was transferred to new tubes. Protein concentration was measured using a spectrophotometer (Bio-Rad) by Bradford assay using Bio-Rad protein assay dye. Samples containing equal protein concentration were then mixed with equal volume 2X SDS Sample Loading Buffer [200 mM Tris-HCl pH 6.8, 20% glycerol, 4% (w/v) SDS, 0.1% Bromophenol blue, 200 mM β-Mercaptoethanol]. Samples were then boiled for 10 mins at 95 degrees Celsius and spun briefly at 13,000 rpm. Samples were then loaded onto SDS-PAGE gel and subsequently transferred to PVDF membrane overnight at 4 degrees Celsius. Membranes were blocked in 5% evaporated milk dissolved in 1X TBST, and immunoblotting was performed as previously described (Zhang et al. 1996; Yang et al. 2002) using primary antibodies against FASN (1:1000) (BD Biosciences; 610962), p65 (1:300) (Santa Cruz; sc-8008), PARP1 (1:1000) (Cell Signaling; 9542), P-p65-Ser536 (1:1000) (Cell Signaling; 93H1), β-Actin (1:5000) (Sigma; A5316), M2-FLAG (1:1000) (Sigma; F1804), Pin1 (1:1000) (Cell Signaling; 3722), and ubiquitin (1:1000) (Cell Signaling; 3933). Images were developed using ECL Western Blotting Detection Reagent (GE Healthcare) on X-Ray film. Images were quantified using ImageJ software.

2.3. Real-Time RT-PCR Analysis

Quantitative real-time PCR analysis was performed as described previously (Dong et al. 2005; Liu et al. 2013). Briefly, cells were cultured in 6-well plates for 48 hours if no drug treatment or 72 hours with drug treatment, wherein RNA was isolated from cell lysate using PureLink RNA Mini Kit (Invitrogen). RNA concentration was measured using a NanoDrop spectrophotometer. RNA was reverse-transcribed to cDNA using equal amounts of RNA for each sample with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) followed by utilization of cDNA for real-time RT-PCR analysis with SYBR Green PCR Mix (Applied Biosystems) using a Step One Plus 7500 Real-Time PCR System (Applied Biosystems). C_T values for target genes were normalized to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Calculations were performed using the comparative C_T method (Schmittgen and Livak 2008). Fold changes between groups were calculated by first normalizing C_T values of target genes to GAPDH C_T values indicating ΔC_T values for each sample. Next, the calculation $2^{-\Delta C_T}$ was performed, and the results of that calculation were used directly to determine fold change between treatment groups or genetic manipulations. Primer sequences utilized for target genes p65 and TNF-α are listed in **Table 4**.

Gene	Target Sequence
GAPDH Forward	AAGGACTCATGACCACAGTCCAT
GAPDH Reverse	CCATCACGCCACAGTTTCC
p65 Forward	TCTCCCTGGTCACCAAGGAC
p65 Reverse	TCATAGAAGCCATCCCGGC
TNF-α Forward	CCCAGGCAGTCAGATCATCTTC
TNF-α Reverse	GGTTTGCTACAACATGGGCTACA

Table 4. Real-time RT-PCR primer sequences.
List of real-time RT-PCR primer sequences for various gene targets investigated in this thesis.

2.4. NF-κB *Cis*-reporter Activity Assay

NF-κB *cis*-reporter assay was performed according to the manufacturer's instructions utilizing the Path Detect *cis*-reporting system (Agilent) as described previously (Wu et al. 2016). Briefly, cells were cultured overnight, followed by transfection with pNF-κB *cis*-reporter plasmid along with co-transfection of pRL-TK renilla luciferase plasmid as an internal control for transfection efficiency using Metafectene Pro Reagent (Biontex). An empty vector excluding the NF-κB reporter elements was also transfected as a negative control for reporter activity. At 48 hours following transfection, cells were harvested and Dual-Luciferase Assay System (Promega) was performed. NF-κB activity was measured using Firefly luciferase measurement and was normalized to renilla luciferase values to account for transfection efficiency.

2.5. ^{35}S-Methionine Pulse-Chase Assay

To pulse-label cells, M3K/shFASN and M3K/shScr control cells were cultured to 75% confluence, then subsequently cultured in methionine-free

DMEM medium supplemented with 10 μCi/mL ^{35}S-Methionine for 2 hours allowing for radiolabeled methionine amino acid incorporation into newly synthesized peptides. ^{35}S-Methionine was then washed out from the culture medium, and cells were cultured in complete DMEM for a chase period of 0-32 hours, wherein cells were harvested at 8 hour intervals and stored at -80 degrees Celsius for later assay. Cell pellets were lysed in TNN lysis buffer, and protein concentration was measured as described above in section **2.2**. 400 μg total protein was then used to immunoprecipitate p65 from total cell protein using an anti-p65 monoclonal antibody (Santa Cruz) and Protein G PLUS-Agarose beads (Santa Cruz). Beads were then washed in lysis buffer to remove off-target proteins. Proteins were eluted from beads by boiling in 2X SDS sample loading buffer, and SDS-PAGE was performed. Gels were dried using a vacuum system gel dryer and exposed to X-Ray film followed by quantification of radioactivity using scintillation counting. Curves for protein degradation were generated using GraphPad Prism 7 to obtain protein half-life measurements based on an average of three independent experiments by curve-fitting using one-phase exponential decay.

2.6. siRNA Transfection

Cells were plated in 6-well plates and cultured for 24 hours. Cells were then transfected using scrambled control siRNA (Cell Signaling) or siRNA against Pin1 (Santa Cruz) in Opti-MEM reduced serum media (Thermo Fisher) using Lipofectamine RNAiMAX reagent (Thermo Fisher) according to the

manufacturer's instructions. 48 hours following transfection, cells were harvested, and cell pellets were stored at -80 degrees Celsius for later assay. Western blot analysis was subsequently performed to verify siRNA knockdown and determine effects on total p65 protein level.

2.7. Cycloheximide Chase Assay

Cells were plated in 6-well plates and cultured for 24 hours to reach 70% confluence. Cells were then transfected following 24 hour cell growth with either pcDNA(3.1) empty vector (negative control), pcDNA(3.1)-p65-FLAG, or pcDNA(3.1)-p65-T254A-FLAG in Opti-MEM reduced serum media using Lipofectamine 3000 Reagent (Thermo Fisher) according to the manufacturer's instructions. WT pcDNA(3.1)-p65-FLAG was generously provided by Dr. Tao Lu, and T254A pcDNA(3.1)-p65-FLAG was generated using site-directed mutagenesis with pcDNA(3.1)-p65-FLAG being utilized as a template. 24 hours following transfection, cells were treated with 60 μg/mL cycloheximide (Sigma) in full serum DMEM media. Cells were then incubated at 37 degrees Celsius for a period of 0 to 12 hours and harvested at the indicated time points. Cell pellets were then stored at -80 degrees Celsius, and Western blot analysis was subsequently performed.

2.8. Ubiquitination Assay

Cells were plated in 6-well plates and cultured for 24 hours to reach 70% confluence. After 24 hours, cells were transfected with WT pcDNA(3.1)-p65-

FLAG using Opti-MEM reduced serum media and Lipofectamine 3000 transfection reagent (Thermo Fisher) according to the manufacturer's instructions. 24 hours following transfection, cells were treated with 2 μM MG132 for a period of 4 hours. Following 4 hours proteasome inhibition, cells were harvested, cell pellets were spun down at 13,000 rpm for 10 minutes at 4 degrees Celsius and stored at -80 degrees Celsius. Cells were subsequently lysed as described earlier in section **2.2** in TNN lysis buffer with intermittent vortexing and incubation on ice. Protein concentration was measured using Bradford assay, 50-100 μg total protein was added equally for paired isogenic cell lines to a microcentrifuge tube, and tube volume was adjusted to 500 μL using TNN buffer. Normal mouse IgG (Santa Cruz) was utilized to pre-clear protein lysates. Soluble protein was then transferred to new microcentrifuge tubes, and 1.5 μg of M2-FLAG antibody (Sigma) was then added to each tube, wherein tubes were placed on a rotator at 4 degrees Celsius for a period of 3 hours to immunoprecipitate p65-FLAG from total protein lysate. After 3 hours immunoprecipitation, 40 μL Protein G-PLUS Agarose beads (Santa Cruz) was added to each tube, and tubes were placed on a rotator at 4 degrees Celsius overnight to conjugate bound p65-FLAG to the beads. Beads were subsequently spun at 5,000xg for 1 minute at 4 degrees Celsius and washed 5 times with TNN buffer containing 1 mM PMSF and 1 mM DTT to remove unbound and off-target proteins. Remaining TNN buffer was removed following the final wash, and proteins were eluted by addition of 2X SDS Sample Loading Buffer and boiling at 95 degrees Celsius for 10 minutes. Beads were spun at 13,000 rpm for 3 minutes

to pellet the beads. Eluted proteins were loaded onto an SDS-PAGE gel. Following SDS-PAGE electrophoresis, gels were transferred to PVDF membranes (Bio-Rad) overnight at 4 degrees Celsius. Membranes were probed using a ubiquitin antibody (Cell Signaling) and exposed to X-Ray film. Western blot analysis was also performed in tandem using input controls to account for similar loading in the immunoprecipitation steps from total protein lysate. Input control blots were probed using antibodies for total p65 (Santa Cruz) and β-Actin (Cell Signaling).

2.9. Cell Viability Assay

Cell viability was measured using the methylene blue cell staining assay. Cells were plated in 96-well plates at $2x10^4$ cells per well and cultured for 24 hours. Media was changed containing cerulenin at varying concentrations using serial dilution or vehicle, and cells were treated for 72 hours. After 72 hour treatment, cells were fixed in 100% methanol for 30 mins at room temperature. After fixing, methanol was aspirated from cells, and 1% (w/v) methylene blue in 10 mM borate buffer, pH 8.5 was added to stain live cells for 30 mins at room temperature. Methylene blue stain was then aspirated from the plates, and cells were subsequently washed three times with 10 mM borate buffer, pH 8.5 to remove excess stain. 0.1 M HCl : 100% ethanol (1:1 w/v) was then added to wells. Plates were shaken to release dye, and absorbance values were read using a plate reader at 650 nm. Data was analyzed using Graph Pad Prism 7 by

subtracting background absorbance values and fitting sigmoidal curve based on the log value of the varying concentrations of cerulenin used in the assay.

2.10. Statistical Analysis

Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey post hoc test where applicable was used for statistical analysis, and $p<0.05$ was considered significant. Results have been presented as mean $\pm$ standard deviation. Statistical calculations were performed using Microsoft Excel and Graph Pad Prism 7. For all statistical calculations for results described herein, at least three independent experiments were performed.

Chapter 3: Results

3.1. Correlation between FASN and total p65 expression in breast cancer cell lines

Initially, to begin uncovering the relationship between NF-κB/p65, Western blot analysis was performed in three breast cancer cell lines utilized henceforth in this study to determine levels of FASN and p65 protein. As shown in **Figure 5**, the highest level of FASN protein was found in the doxorubicin-resistant M3K cells, while the lowest level of FASN protein was found in the parental MDA-MB-468 breast cancer cells. Contrarily, for total p65 level, the cell line containing the highest relative protein level was the parental MDA-MB-231 cells, while the M3K cells had the lowest p65 level among the three cell lines tested. This observation indicates a general negative correlation between FASN and total p65 level in breast cancer cells.

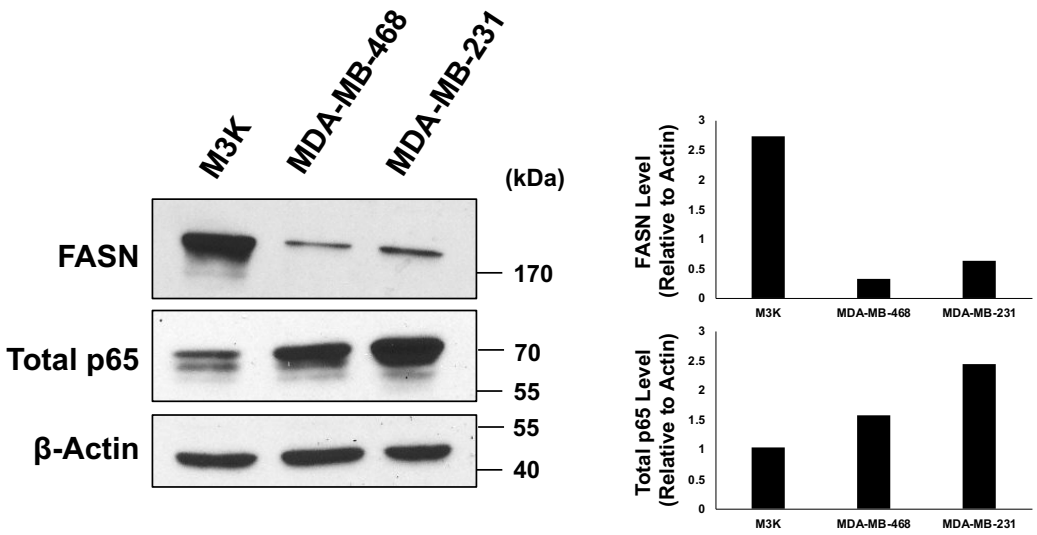


Figure 5. Correlation between FASN and total p65 expression in breast cancer cell lines.

Western blot analysis showing FASN and total p65 protein level in M3K, MDA-MB-468, and MDA-MB-231 cells relative to β-Actin loading control. Images from one experiment were quantified using ImageJ.

3.2. FASN expression negatively regulates total p65 protein level in breast cancer cells

It has previously been shown that FASN exhibits inhibitory action towards NF-κB (Wu et al. 2016). To determine if FASN regulates NF-κB/p65 at the protein level in breast cancer cells, Western blot analysis was performed using a previously established drug resistant cell line with stable FASN knockdown (M3K/shFASN) derived from MCF7 parental breast cancer cells (Liu et al. 2013). I observed that FASN knockdown resulted in an increase in total p65 protein level relative to scrambled shRNA control cells (M3K/shScr) (**Figure 6B**).

To validate the above finding, and to ensure that FASN regulation of p65 expression is not specific to MCF7-derived cells, a stable MDA-MB-436 cell line with FASN overexpression (MDA-MB-436/FASN) was established using transfection of pcDNA(3.1)-FASN and G418 drug selection (**Figure 6A**). The MDA-MB-436 cell line was chosen due to its clinical relevance as representative of the aggressive nature and poor prognosis of the TNBCs. It was also chosen due to its low expression of FASN relative to other breast cancer cell lines as a way to avoid any potential artefacts associated with overexpression of FASN in these cells.

Utilizing this newly established overexpression cell line, I observed that total p65 protein level was decreased when FASN was overexpressed relative to vector-transfected control cells (MDA-MB-436/Vec) (**Figure 6B**). This indicates that there is an inverse relationship in breast cancer cells between FASN and total p65 protein level, wherein FASN negatively regulates total p65 protein level.

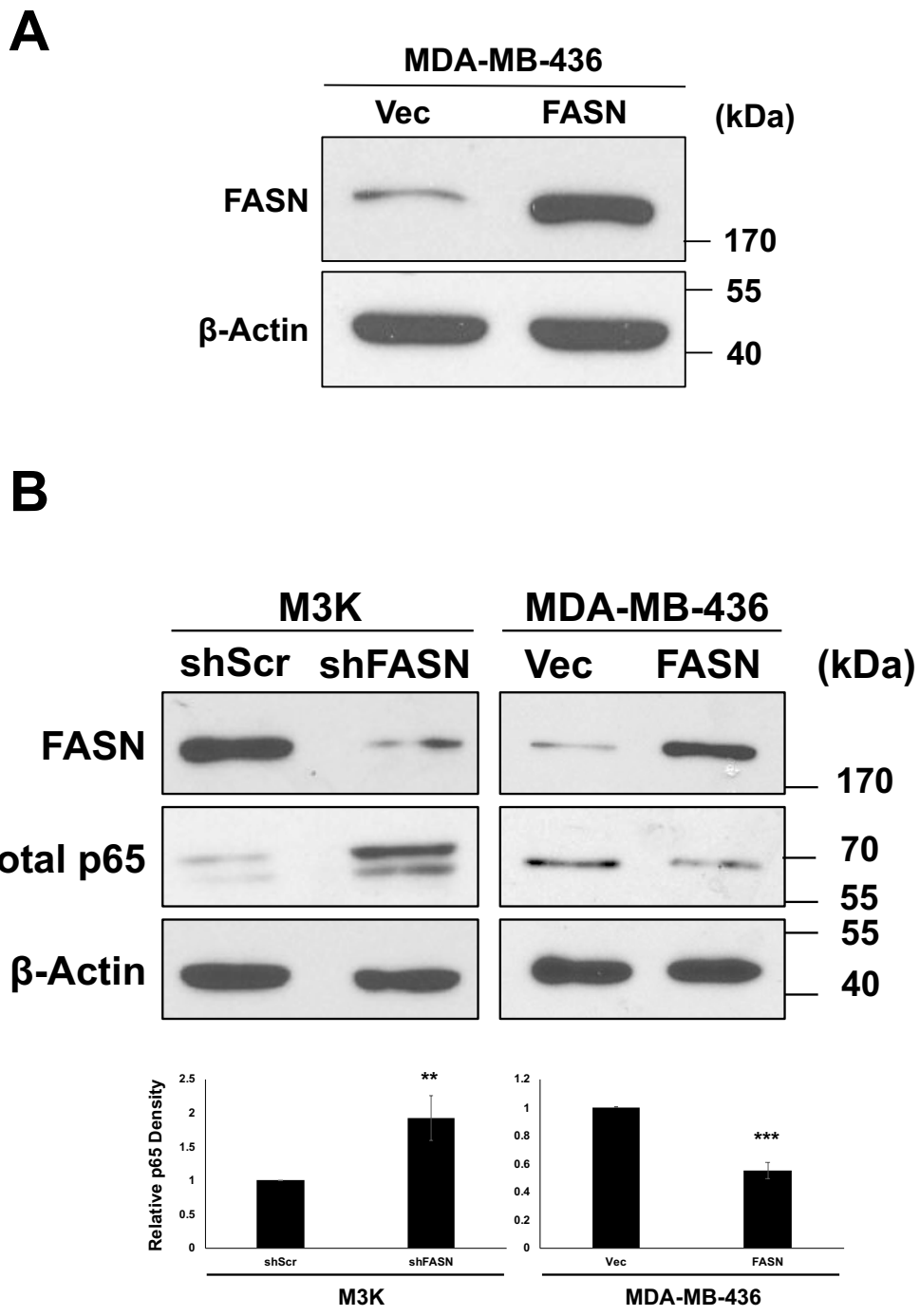


Figure 6. FASN expression negatively regulates total p65 protein level in breast cancer cells.

(A) Western blot verifying and confirming stable FASN overexpression in MDA-MB-436 breast cancer cells (MDA-MB-436/FASN) compared to vector-

transfected control cells (MDA-MB-436/Vec). (B) Western blot analysis using M3K cells with stable FASN shRNA knockdown compared to scrambled shRNA control cells and MDA-MB-436 cells with stable FASN overexpression compared to vector-transfected control cells looking at total p65 protein level. Blots were quantified using ImageJ. Differences in protein level were determined using Student's *t*-test. **p<0.01; ***p<0.001. Bar graphs represent three independent experiments with standard deviations from the mean.

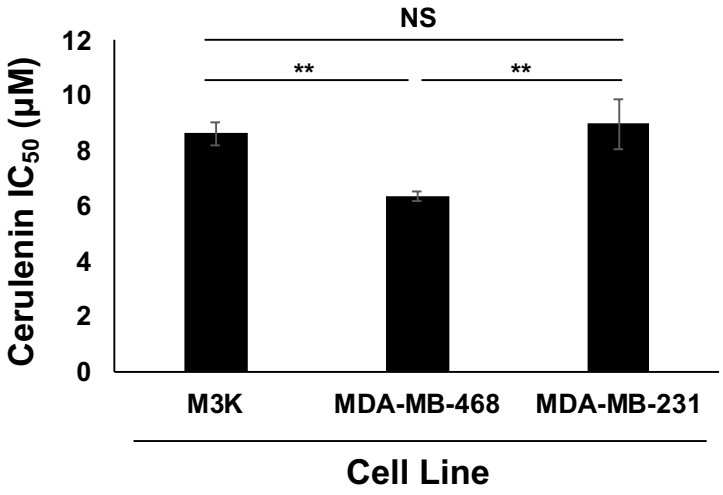
3.3. Pharmacological inhibition of FASN using cerulenin increases total p65 expression in breast cancer cells

Having demonstrated a relationship wherein FASN expression negatively regulates p65 protein level, I sought to determine if FASN activity plays a role in this process. To explore the role of FASN activity in this mechanism, I utilized a common FASN inhibitor known as cerulenin, which binds irreversibly to the β-ketoacyl synthase domain of FASN, thereby preventing the elongation of the growing fatty acid chain via condensation reaction (Vance et al. 1972; Omura 1976; Moche 1999). Initially, in an effort to determine specific concentrations to employ for experimentation in various cell lines, IC_{50} values were determined for cerulenin treatment in the three breast cancer cell lines utilized in this study using the Methylene blue cell viability assay (**Figure 7A**). Similar IC_{50} values were observed for M3K and MDA-MB-231 cells at 8.62 ± 0.39 μM (1.92 ± 0.09 μg/mL) and 8.98 ± 0.90 μM (2.00 ± 0.20 μg/mL) respectively, whereas MDA-MB-468 cells exhibited a significantly lower IC_{50} value of 6.35 ± 0.16 μM (1.42 ± 0.04 μg/mL). Representative curves for cell viability in the presence of varying concentrations of cerulenin for each cell line are shown in **Figure 7B.**

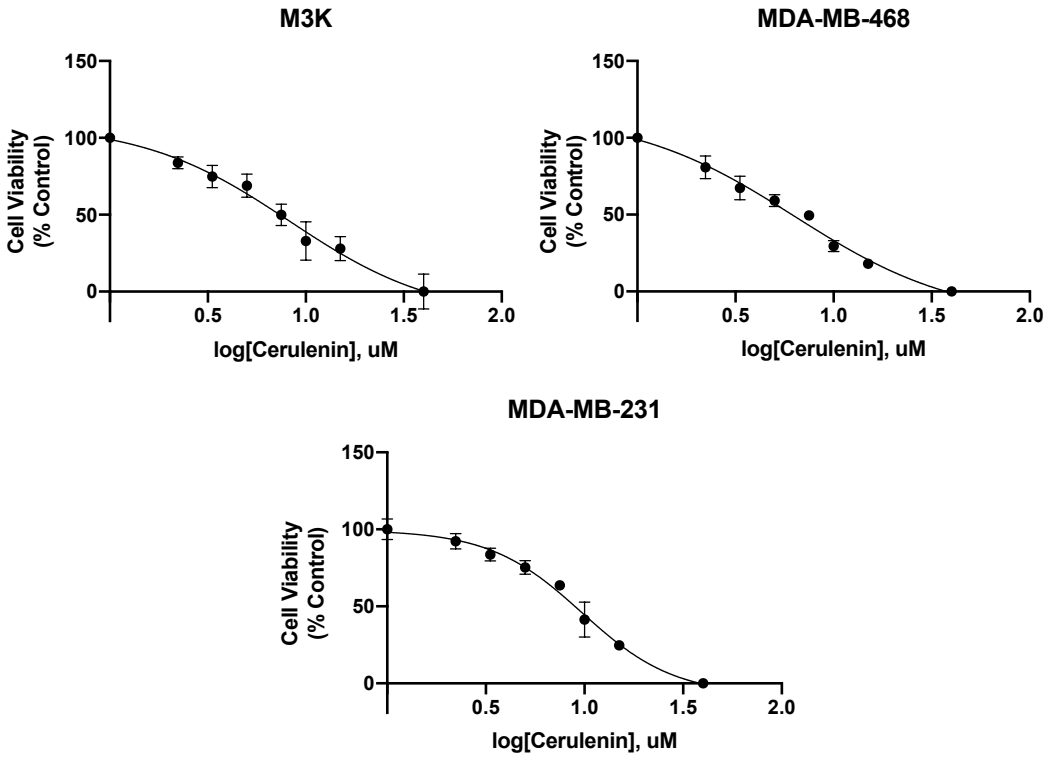
As a result of the IC_{50} data obtained for cerulenin with respect to cell viability in the three cell lines, M3K, MDA-MB-468, and MDA-MB-231 cells were treated for 72 hours with increasing concentrations of cerulenin or vehicle (ethanol), and Western blot analysis was performed for total p65 protein level in these cell lines. In these experiments, a lower starting concentration for cerulenin (1.75 μg/mL) was utilized in MDA-MB-468 cells due to the significantly lower IC_{50}

value that was determined for these cells compared to M3K and MDA-MB-231 cells. With increasing concentrations of cerulenin, there was a resulting dose-dependent increase in total p65 protein level compared to untreated cells in all cell lines tested (**Figure 7C**). It was also found that cerulenin appeared to induce a decrease in FASN expression in all cell lines tested indicating that cerulenin indeed binds to and inhibits FASN and may induce its degradation. Overall, these findings suggest that FASN activity, similar to FASN expression, also exhibits a relationship with NF-κB/p65, wherein FASN negatively regulates total p65 at the protein level.

A


NS
**
**
Cerulenin IC50 (μM)
12
10
8
6
4
2
0
M3K
MDA-MB-468
MDA-MB-231
Cell Line

B


M3K
MDA-MB-468
MDA-MB-231
Cell Viability (% Control)
150
100
50
0
0.5
1.0
1.5
2.0
log[Cerulenin], uM

C

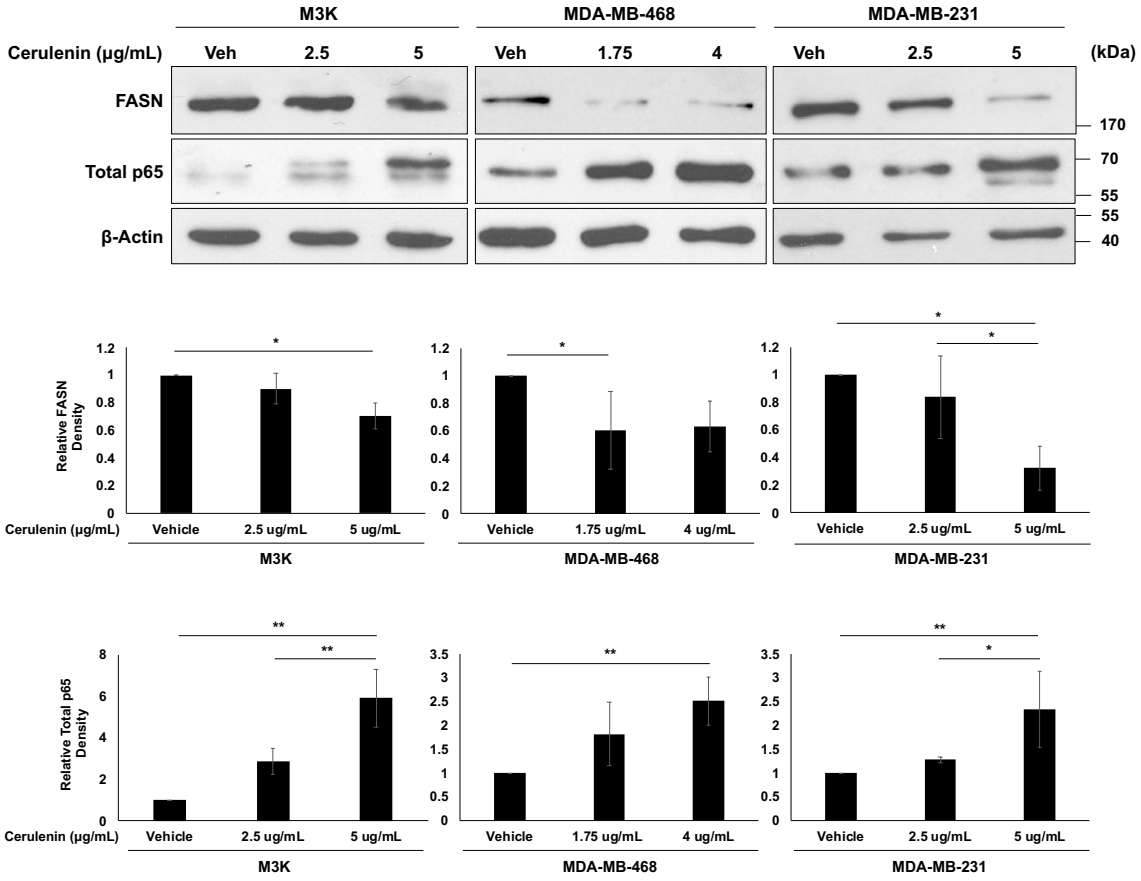


Figure 7. Pharmacological inhibition of FASN using cerulenin increases total p65 expression in breast cancer cells.

(A) Bar graph representation of methylene blue cell viability assay data indicating relative IC_{50} values for M3K, MDA-MB-468, and MDA-MB-231 cells treated with varying concentrations of FASN inhibitor cerulenin or vehicle (ethanol) for 72 hours. (B) Representative cell viability curves for methylene blue assays in M3K, MDA-MB-468, and MDA-MB-231 cells. (C) Western blot analysis using M3K, MDA-MB-468, and MDA-MB-231 cells treated with increasing concentrations of cerulenin or vehicle (ethanol) for 72 hours probing for total p65 and FASN level. Methylene blue assay data was curve-fitted to determine IC_{50} values using Graph

Pad Prism. X-ray film images were quantified using ImageJ. Differences in IC_{50} values between cell lines and protein level in Western blots were determined using one-way ANOVA with Tukey post hoc test. *$p<0.05$; **$p<0.01$. Bar graphs represent three independent experiments, wherein error bars denote standard deviation from the mean.

3.4. Supplementation of breast cancer cells with exogenous palmitate ablates FASN-mediated effects on total p65

As discussed earlier, the end product of FASN enzymatic activity is the 16-carbon saturated fatty acid palmitate. As a result, to further investigate the role of FASN activity in regulating NF-κB/p65, the potential effects of palmitate on NF-κB/p65 were investigated. To this end, M3K/shFASN cells were treated with or without 30 μM exogenous palmitate for a period of 48 hours, followed by Western blot analysis of p65 expression. I observed exogenous palmitate was able to ablate the increases in total p65 protein level that are seen following FASN shRNA knockdown (**Figure 8A**). This finding suggests that palmitate, as the product of FASN activity, may play a specific role in mediating the downstream effects that are able to result in decreased NF-κB/p65 expression in breast cancer cells.

The M3K/shScr control cells without FASN knockdown were also treated with or without exogenous palmitate to investigate the basal effects on total p65 in the absence of FASN knockdown (**Figure 8B**). Palmitate treatment in these cells also reduced total p65 level, which indicates that palmitate is able to suppress p65 expression induced by FASN knockdown and suppress the basal level of p65, suggesting that the ability of palmitate to regulate total p65 level is not dependent on a lower level of endogenous free fatty acids in the cell as the M3K/shScr cells would exhibit higher levels of circulating free fatty acids.

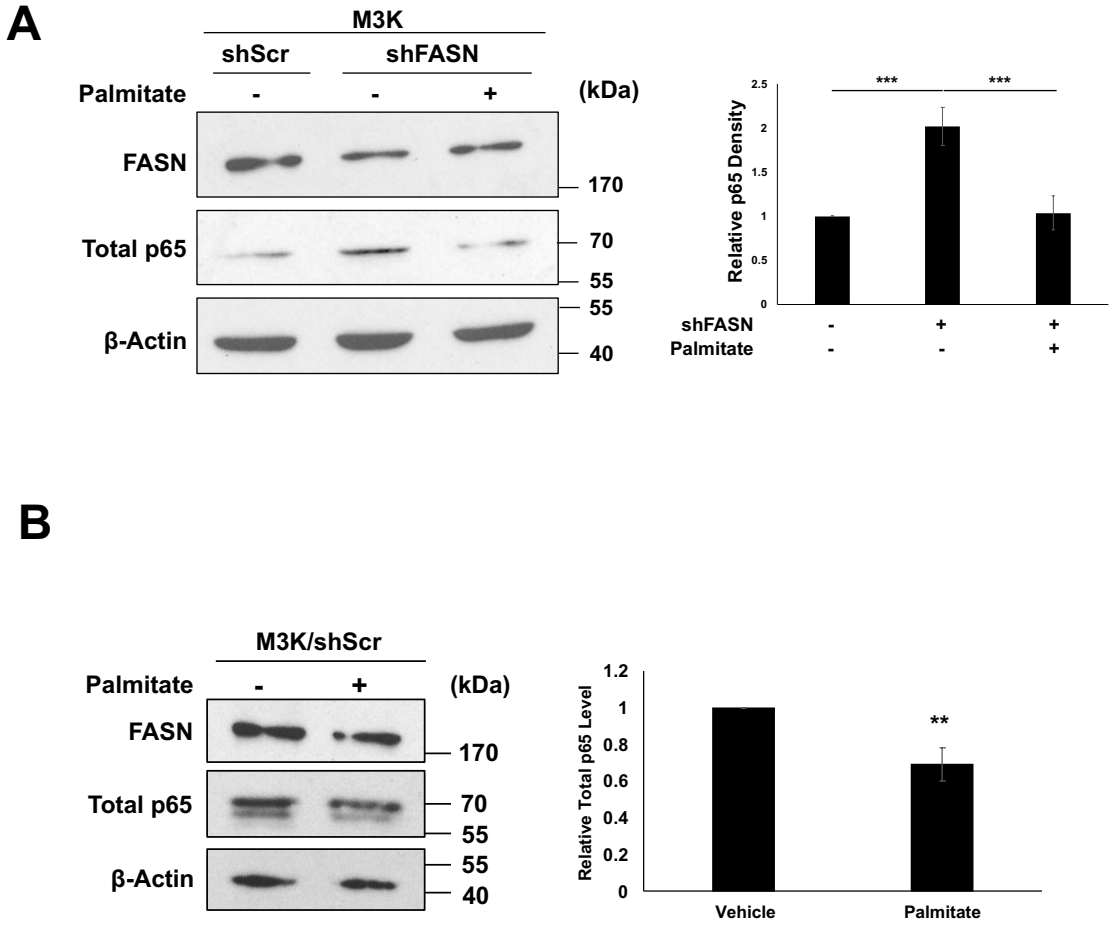


Figure 8. Supplementation of breast cancer cells with exogenous palmitate ablates FASN-mediated effects on total p65.

Western blot analysis wherein M3K/shFASN cells (A) or M3K/shScr control cells (B) were treated with vehicle (DMSO) or 30 μM exogenous palmitate for 48 hours. Cells were then assayed for total p65 expression by probing blots with a total p65 antibody. Images were quantified using ImageJ. Significant differences were determined using one-way ANOVA with Tukey post hoc test. **p<0.01; ***p<0.001. Bar graphs with error bars represent three independent experiments with standard deviation from the mean.

3.5. FASN expression does not affect total p65 at the transcriptional level

In order to investigate the potential role of p65 transcriptional effects in the ability of FASN expression changes to negatively regulate NF-κB/p65 at the protein level, the effects of FASN expression changes on total p65 mRNA were examined. Real-time RT-PCR analysis was performed using the M3K/shFASN cells along with their control cells, as well as the MDA-MB-436/FASN cells along with their control cells following 48 hours cell growth in 6-well plates. Both FASN knockdown and FASN overexpression had no significant effect on total p65 mRNA level relative to their respective control cells (**Figure 9**). This finding suggests that, while FASN has the ability to negatively regulate NF-κB/p65 protein expression, this regulation does not occur at the transcriptional level.

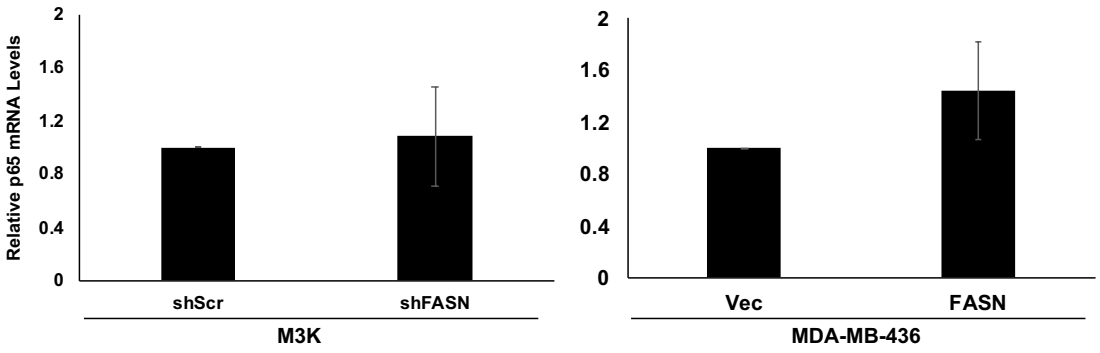


Figure 9. FASN expression does not affect total p65 at the transcriptional level. Real-time RT-PCR analysis using M3K cells with stable FASN shRNA knockdown compared to scrambled shRNA control cells and MDA-MB-436 cells with stable FASN overexpression compared to vector-transfected control cells measuring total p65 mRNA level normalized to GAPDH mRNA level (housekeeping gene). Potential differences in mRNA levels were determined using Student's *t*-test. Bar graphs represent three independent experiments with standard deviation from the mean.

3.6. FASN pharmacological inhibition does not affect total p65 at the transcriptional level

Having shown that FASN expression changes do not result in an effect on p65 mRNA level, I wanted to subsequently investigate the potential effects of FASN pharmacological inhibition on total p65 mRNA levels in order to determine whether FASN inhibition likely affects total p65 through a mechanism similar to that of FASN expression changes. To this end, M3K, MDA-MB-468, and MDA-MB-231 parental breast cancer cells were treated for 72 hours with cerulenin at 5 μg/mL, 2.5 μg/mL, or 5 μg/mL respectively or vehicle (ethanol) for a period of 72 hours. Total RNAs were then isolated from these cells and real-time RT-PCR analysis was performed to determine the effects of cerulenin treatment on total p65 mRNA. I observed in all three cell lines tested that cerulenin treatment resulted in no significant effect on total p65 mRNA levels (**Figure 10**). This finding indicates that the effects of FASN pharmacological inhibition on total p65 protein level are not a result of effects on the transcription of p65. Also, taken together with the lack of a significant effect of FASN expression changes on p65 mRNA level, this finding suggests that FASN regulation of NF-κB occurs at the protein level, potentially impacting either the rate of p65 translation or the rate of protein turnover of p65.

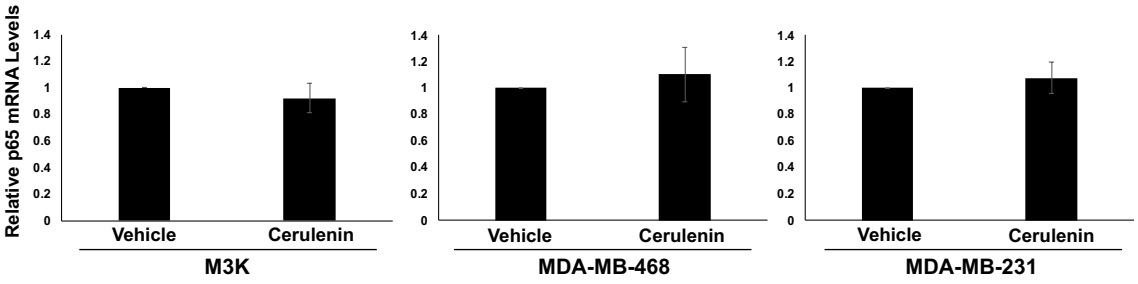


Figure 10. FASN pharmacological inhibition does not affect total p65 at the transcriptional level.

M3K cells, MDA-MB-468 cells, or MDA-MB-231 cells were treated with 5 μg/mL, 2.5 μg/mL, or 5 μg/mL cerulenin respectively or vehicle (ethanol) for 72 hours followed by real-time RT-PCR analysis measuring total p65 mRNA normalized to GAPDH as an internal control. Potential differences in mRNA levels between treatment groups were determined using Student's *t*-test. Bar graphs represent three independent experiments with error bars describing standard deviation from the mean.

3.7. FASN expression negatively regulates NF-κB activity in breast cancer cells

Having shown that FASN expression and activity negatively regulates the expression of NF-κB/p65, I next sought to determine whether the effects of FASN on NF-κB also correlated with an effect on NF-κB activity in addition to the effects on p65 at the level of expression. To investigate the relationship between FASN and NF-κB activity, I utilized a commercially available luciferase reporter assay system that uses a plasmid containing consensus NF-κB binding elements, wherein the ability of NF-κB to bind to target gene promoters to carry out its transcriptional activity is measured by the induction of luciferase gene expression.

At 48 hours after transfection of the NF-κB reporter plasmid into M3K/shFASN and M3K/shScr control cells, luciferase expression was measured as an indicator of NF-κB activity. As shown in **Figure 11**, when FASN was knocked down, an increase in NF-κB activity was observed relative to scrambled shRNA control cells. Contrarily, NF-κB activity decreased in MDA-MB-436 cells with FASN overexpression compared to the MDA-MB-436/Vec control cells. These results are consistent with the observed downregulation of p65 by FASN.

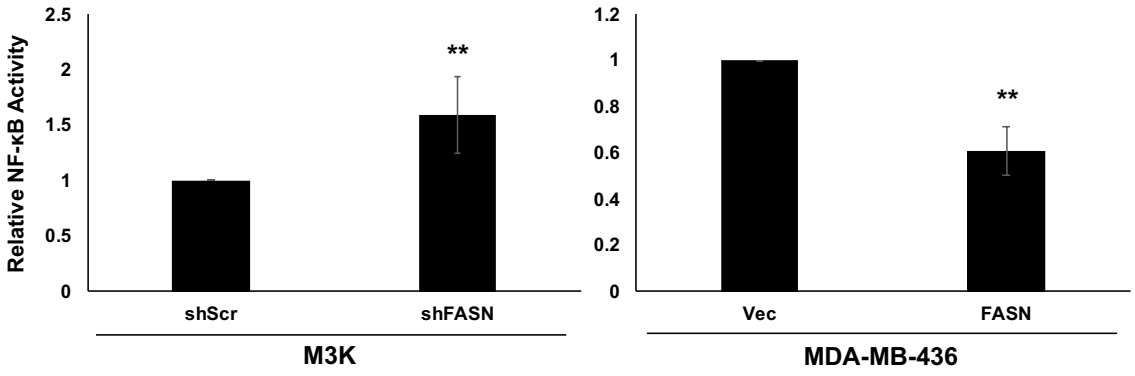


Figure 11. FASN expression negatively regulates NF-κB activity in breast cancer cells.

M3K/shFASN cells along with M3K/shScr control cells or MDA-MB-436/FASN cells along with MDA-MB-436/Vec control cells were transfected for 48 hours with an NF-κB *cis*-reporter plasmid containing the firefly luciferase gene, along with a pRL-TK renilla luciferase plasmid as an internal control for transfection efficiency. After 48 hours transfection, dual luciferase assay was performed to measure luciferase gene expression as an output of NF-κB activity. Potential differences in NF-κB activity between cell line pairs was determined using Student's *t*-test. **p<0.01. Bar graphs represent three independent experiments, wherein error bars represent standard deviation from the mean.

3.8. FASN expression negatively regulates TNF-α expression in breast cancer cells

To confirm the effects of FASN on NF-κB activity, I investigated the effects of FASN expression changes on NF-κB target gene expression. Previous research in the Zhang lab showed a potential relationship between FASN and the canonical NF-κB target gene TNF-α (Liu et al. 2013). As a result, the potential effects of FASN expression changes on TNF-α gene expression were investigated using real-time RT-PCR analysis with M3K/shFASN cells and M3K/shScr control cells, as well as MDA-MB-436/FASN cells and MDA-MB-436/Vec control cells. RT-PCR analysis showed that FASN shRNA knockdown in M3K cells resulted in an increase in TNF-α mRNA level relative to scrambled shRNA control cells, whereas overexpression of FASN in MDA-MB-436 cells resulted in a decrease in TNF-α mRNA levels relative to vector control cells (**Figure 12**). These results indicate that, similar to the effects of FASN on p65, FASN expression negatively regulates TNF-α expression as a downstream target gene of NF-κB.

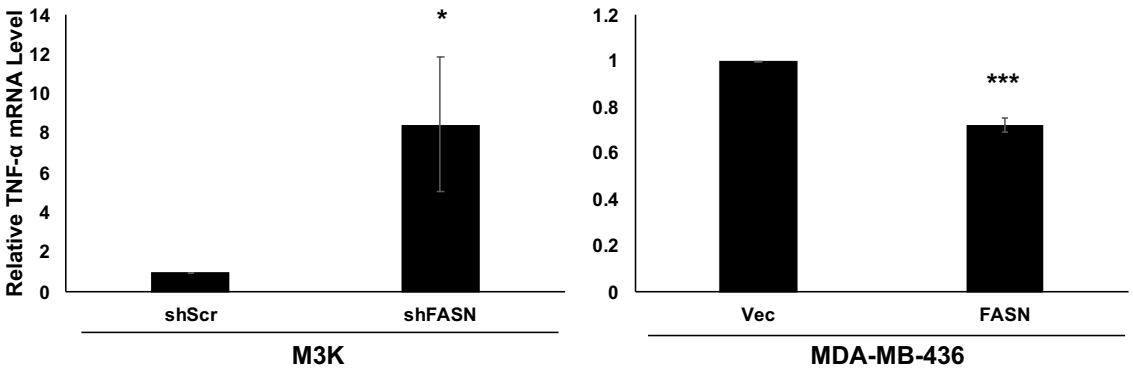


Figure 12. FASN expression negatively regulates TNF-α expression in breast cancer cells.

Real-time RT-PCR analysis in M3K/shFASN cells compared to M3K/shScr control cells and MDA-MB-436/FASN cells compared to MDA-MB-436/Vec control cells looking at TNF-α mRNA levels normalized to GAPDH mRNA levels (housekeeping gene) shown as relative expression level to control. Potential significant differences were determined using Student's *t*-test. *p<0.0.5; ***p<0.001. Bar graphs represent three independent experiments. Error bars represent standard deviation from the mean.

3.9. FASN expression positively regulates PARP1 expression in breast cancer cells

To further confirm the effects of FASN on NF-κB activity, I investigated the relationship between FASN and PARP1, a second gene correlated with NF-κB. As discussed earlier, PARP1 was implicated in the FASN signaling mechanism, wherein FASN induces increases in NHEJ DNA repair activity in cancer cells to promote cell survival in the presence of chemotherapeutic and other DNA-damaging agents. In this mechanism, p65 was shown to negatively regulate PARP1 transcription in breast and pancreatic cancer cells, and, specifically, this occurred via a composite transcription factor binding site shared with another common transcription factor, SP1 (Wu et al. 2016).

To verify that PARP1 is positively regulated in breast cancer cells downstream of FASN, the effects of FASN expression changes on PARP1 protein level were measured by Western blot. As shown in **Figure 13**, when FASN was knocked down in M3K cells, there was a resulting decrease in PARP1 protein level relative to scrambled shRNA control cells. On the other hand, when FASN was overexpressed in MDA-MB-436 cells, this led to a corresponding increase in PARP1 protein level compared to vector control cells.

This suggests that FASN indeed does positively regulate PARP1 expression in correlation with the negative regulation of NF-κB/p65 in breast cancer cells. Taken in context with the effects of FASN on NF-κB reporter activity, as well as the effects of FASN on the NF-κB target gene TNF-α, this result also indicates that FASN negatively regulates NF-κB activity.

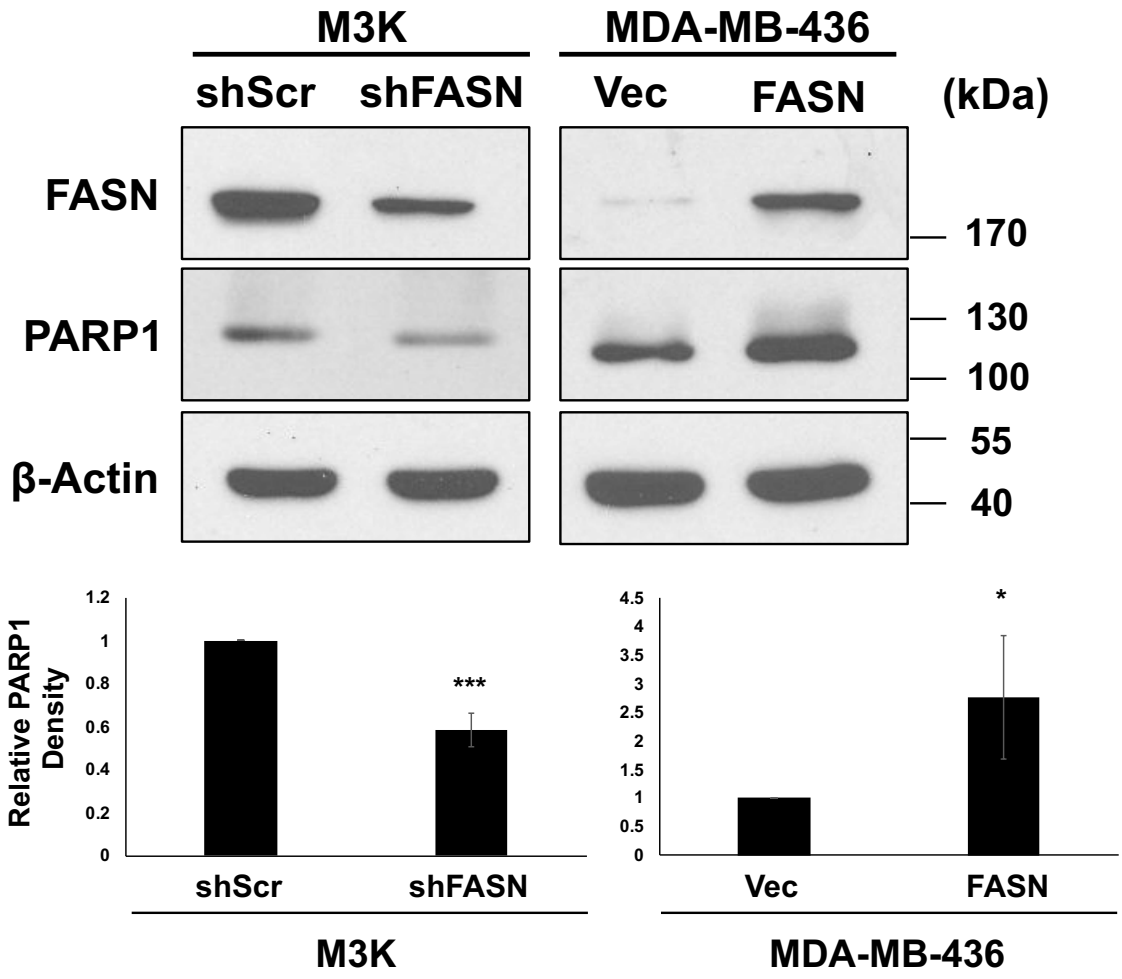


Figure 13. FASN expression positively regulates PARP1 expression in breast cancer cells.

Western blot analysis using M3K/shFASN cells and M3K/shScr control cells, as well as MDA-MB-436/FASN cells and MDA-MB-436/Vec control cells probing for PARP1 protein. X-ray film images were quantified using ImageJ. Potential significant differences between groups were determined using Student's *t*-test. *p<0.05; ***p<0.001. Bar graphs are a representation of three independent experiments, wherein error bars indicate standard deviation from the mean.

3.10. FASN negatively regulates p65 protein stability

Thus far, the results in this thesis have demonstrated that FASN expression and activity negatively regulates p65 at the protein level but does not affect NF-κB/p65 transcription. As a result, I next sought to determine the mechanism responsible for the negative effects exerted by FASN on p65 protein. To this end, I hypothesized that FASN may impact p65 protein stability.

To investigate the potential role of FASN in regulating p65 protein stability, M3K/shFASN cells or M3K/shScr control cells were utilized in a pulse-chase assay, wherein cells were harvested at 8 hour intervals for a total chase period of 32 hours following washout of radiolabeled ^{35}S-Methionine. Immunoprecipitation for total p65 and SDS-PAGE was performed using the harvested protein to determine any potential differences in the rate of protein turnover between the two cell lines, and the precipitated p65 was subsequently quantified using scintillation counting.

As shown in **Figure 14**, total p65 protein decreased at a noticeably faster rate in M3K/shScr cells compared to M3K/shFASN cells. Specifically, when p65 protein half-life was determined using Graph Pad Prism, I observed that FASN knockdown resulted in a substantially higher half-life (19 hours) compared to that in the scrambled shRNA control cells (6 hours). This finding was similar to previously published findings for p65 half-life for WT p65 and a mutated p65 exhibiting decreased protein stability (Ryo et al. 2003). This result suggests that increased FASN expression in breast cancer cells results in decreased p65 protein stability.

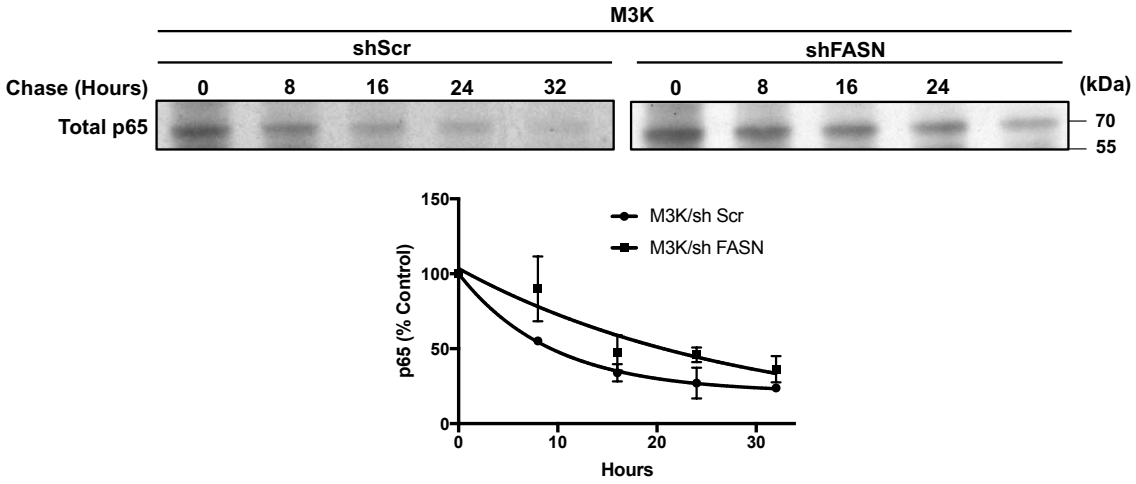


Figure 14. FASN negatively regulates p65 protein stability.

^{35}S-Methionine pulse-chase analysis using M3K/shFASN cells and M3K/shScr control cells. Cells were incubated in the presence of ^{35}S-Methionine for 2 hours prior to washout and incubation in normal media for 32 hours. Cells were harvested every 8 hours for total p65 immunoprecipitation, SDS-PAGE, and Western blot analysis. X-ray film was developed and total p65 bands were quantified using scintillation counting. Protein half-life was determined using Graph Pad Prism, and data was graphed using one-phase exponential decay for curve-fitting. Graph is representative of three independent experiments. Error bars represent standard deviation from the mean.

3.11. FASN induces p65 proteasomal degradation

Having shown that FASN can lead to reduced p65 protein stability and, as a result, an increase in degradation of p65, I next wanted to determine the mechanism behind FASN-mediated degradation of p65. NF-κB/p65 has been shown to be degraded through the proteasomal pathway (Saccani et al. 2004). As a result, I hypothesized that FASN may promote degradation of p65 via the proteasome. As shown in **Figure 15A**, when MDA-MB-436/FASN cells were treated with 2 μM MG132 for 24 hours to block proteasomal degradation of proteins, the ability of FASN to reduce p65 protein level was ablated, and a rescue in p65 protein level was observed.

In order to investigate the effects of proteasome inhibition on total p65 level in breast cancer cells at the basal level, MDA-MB-436/Vec control cells were treated with 2 μM MG132 or vehicle for 24 hours before Western blot analysis was performed. As shown in **Figure 15B**, though there appeared to be a slight trend towards an increase in total p65 level with MG132 treatment in MDA-MB-436/Vec cells, this trend was not significant compared to vehicle-treated cells. This finding suggests that the rescue effect on total p65 level that was seen with proteasome inhibition in **Figure 15A** is in response to the loss in total p65 level driven by increases in FASN expression rather than a global basal effect on p65 level resulting from consistent degradation of the protein.

Next, a previously generated MCF7 cell line with stable FASN overexpression (MCF7/FASN) (Liu et al. 2008) was utilized to confirm that blocking proteasomal degradation with MG132 prevents the ability of FASN to

reduce p65 protein level. As shown in **Figure 15C**, when MCF7/FASN cells were treated with 2 μM MG132 for 24 hours, a rescue in p65 protein level was observed relative to vehicle-treated cells. Taken together, these results suggest that a high level of FASN expression promotes the degradation of p65 through the proteasome, and that blocking proteasomal degradation can rescue FASN downregulation of protein.

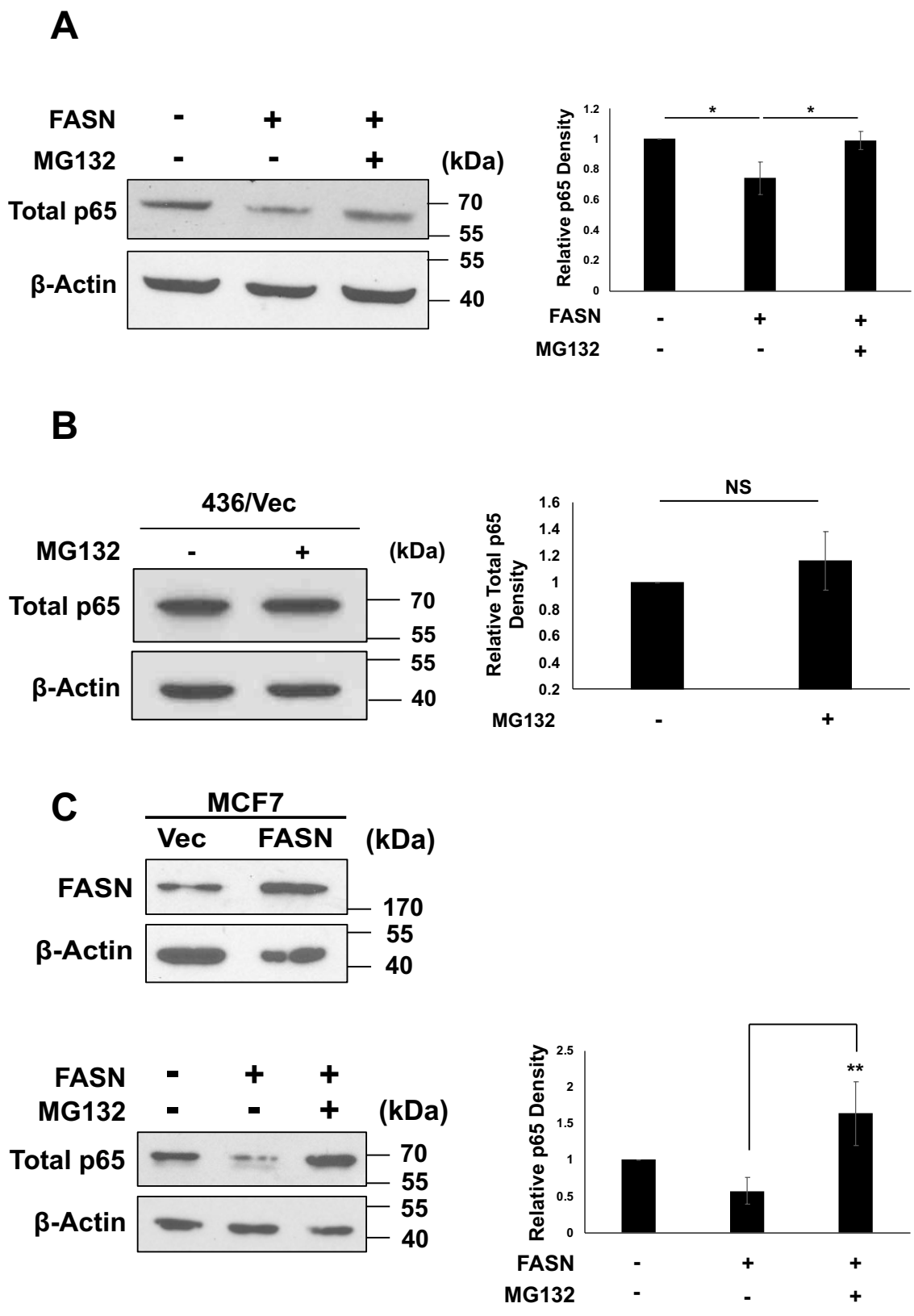

A
FASN - + +
MG132 - - +
(kDa)
Total p65
70
55
β-Actin
55
40
Relative p65 Density
1.2
1
0.8
0.6
0.4
0.2
0
*
*
FASN - + +
MG132 - - +
B
436/Vec
MG132 - +
(kDa)
Total p65
70
55
β-Actin
55
40
Relative Total p65 Density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
NS
MG132 - +
C
MCF7
Vec FASN
(kDa)
FASN
170
β-Actin
55
40
FASN - + +
MG132 - - +
(kDa)
Total p65
70
55
β-Actin
55
40
Relative p65 Density
2.5
2
1.5
1
0.5
0
**
FASN - + +
MG132 - - +

Figure 15. FASN induces p65 proteasomal degradation.

Western blot analysis of (A) MDA-MB-436/FASN cells, (B) MDA-MB-436/Vec control cells, and (C) MCF7/FASN cells treated with 2 μM MG132 for 24 hours compared to cells treated with vehicle (DMSO) for total p65. X-ray film images were quantified using ImageJ. Potential significant differences were determined using one-way ANOVA with Tukey post hoc test or Student's *t*-test. *$p<0.05$; **$p<0.01$. Bar graphs represent three independent experiments. Error bars delineate standard deviation from the mean.

3.12. FASN induces ubiquitination of p65

Having seen that increases in FASN expression result in increased proteasomal degradation of p65, I wanted to determine whether FASN expression could also impact ubiquitination of p65 as a signal for the cell to trigger proteasomal degradation, as proteasomal degradation of p65 has been heavily linked to p65 ubiquitination by a variety of different E3 ubiquitin ligases (Collins et al. 2016).

In order to investigate ubiquitination levels of p65 with changes in FASN expression, M3K/shFASN cells along with M3K/shScr control cells, as well as MDA-MB-436/FASN cells along with MDA-MB-436/Vec control cells were initially transfected with pcDNA(3.1)-p65-FLAG for 24 hours, followed by 4 hour treatment with 2 μM of the proteasome inhibitor MG132. MG132 treatment was used to prevent any proteasomal degradation of p65-FLAG proteins that would occur following ubiquitination of those proteins. As a result, this will allow for an accurate representation of the total level of ubiquitinated-p65-FLAG that is seen downstream of FASN expression changes.

After harvesting cells, p65-FLAG proteins were then immunoprecipitated, and Western blot analysis was performed using a ubiquitin antibody to measure the level of ubiquitination of p65-FLAG proteins. Western blot analysis was also performed using total cell lysate to measure levels of p65-FLAG prior to immunoprecipitation in the presence of MG132 to prevent degradation of FLAG-p65 proteins, as well as to measure levels of β-actin in total cell lysate as a control to attempt to ensure equal amounts of protein utilized in the

immunoprecipitation of p65-FLAG. Also, to control for possible transfection efficiency differences in ectopic expression of p65-FLAG between cell lines, quantification was performed using ImageJ, wherein the ratio of ubiquitinated-p65-FLAG levels to input level of p65-FLAG was determined.

As shown in **Figure 16**, M3K/shFASN cells exhibited decreased ubiquitination of p65-FLAG compared to M3K/shScr control cells. On the contrary, when FASN was overexpressed in MDA-MB-436 cells, p65-FLAG ubiquitination levels were increased compared to MDA-MB-436/Vec control cells. This finding, in correlation with the effects of FASN on proteasomal degradation, suggests that increased FASN expression results in increased p65 ubiquitination.

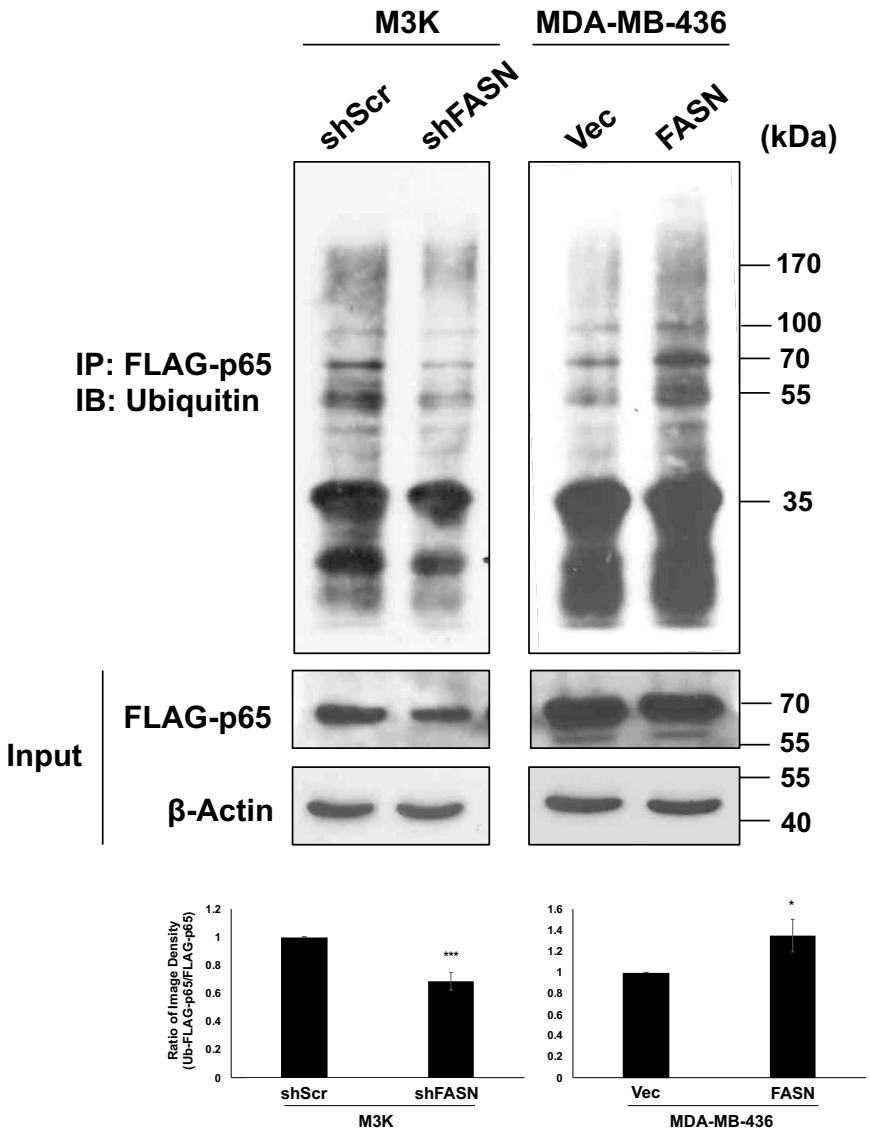


Figure 16. FASN induces ubiquitination of p65.

Western blot analysis of p65-FLAG protein immunoprecipitated from M3K/shFASN cells or M3K/shScr control cells, as well as MDA-MB-436/FASN cells or MDA-MB-436/Vec control cells transfected with p65-FLAG for 24 hours followed by treatment with 2 μM MG132 for 4 hours. Western blots were probed with an α-ubiquitin antibody. Total protein lysate was subjected to Western blot for input levels as a loading control, wherein Western blots were probed for p65-

FLAG and β-actin. Images are representative of three independent experiments and were quantified using ImageJ. Potential significant differences in ubiquitinated-p65-FLAG were determined using Student's *t*-test. *$p<0.05$; ***$p<0.001$. Error bars represent standard deviation from the mean.

3.13. Kinase inhibition reduces p65 protein level

The data presented in this thesis to this point shows that FASN negatively regulates p65 by decreasing its stability, likely through proteasomal degradation. Therefore, I next sought to determine the mechanism responsible for the disruption in p65 protein stability mediated by FASN. Previous evidence suggests that phosphorylation of certain sites on the p65 subunit of NF-κB, including Thr254, Ser276, and Ser536 are involved in promoting the activation of the NF-κB signaling pathway and may be involved in regulating p65 protein stability (Christian et al. 2016).

As a result, I sought to investigate the potential role of kinase activity in mediating the effects of FASN on p65 protein. To this end, M3K/shFASN cells were treated with or without the pan-kinase inhibitor staurosporine, which binds to the ATP binding pocket of kinases, at a concentration of 100 nM for 2 hours followed by stimulation with 50 μg/mL TNF-α for 1 hour, wherein cells were harvested and Western blot analysis was performed to measure the effects of kinase inhibition with staurosporine on total p65 level and on levels of phosphorylation of p65 at Ser536. TNF-α stimulation was used specifically in an effort to induce detectable levels of phosphorylation of p65 at Ser536, which was measured to control for effective kinase inhibition and to investigate a potential link between FASN and p65 phosphorylation. TNF-α was chosen to stimulate NF-κB in this context, as opposed to other mediators of NF-κB activation, due to its established relationship with FASN. In a previous study, it was observed that, in both the presence and absence of doxorubicin treatment to induce DNA

damage, knockdown of FASN expression in breast cancer cells resulted in an upregulation of TNF-α expression and promoter activity, as well as NF-κB activity (Liu et al. 2013). In this study, FASN overexpression also negatively regulated TNF-α expression. As a result, it appears likely that TNF-α is an upstream regulator of NF-κB in the mechanism of NF-κB activation downstream of decreased FASN expression. Western blot analysis was then performed to investigate the effects of staurosporine treatment on total p65 protein.

As shown in **Figure 17**, kinase inhibition ablated the ability of FASN knockdown in M3K breast cancer cells to cause increases in total p65 protein level and subsequently resulted in a reduction in total p65 level to a level similar to that of M3K/shScr control cells. It was also determined that FASN knockdown resulted in an increase in phosphorylation of p65 at Ser536, while kinase inhibition with staurosporine ablated these increases. As a result, there may be a link between FASN, kinase activity, and p65 protein expression.

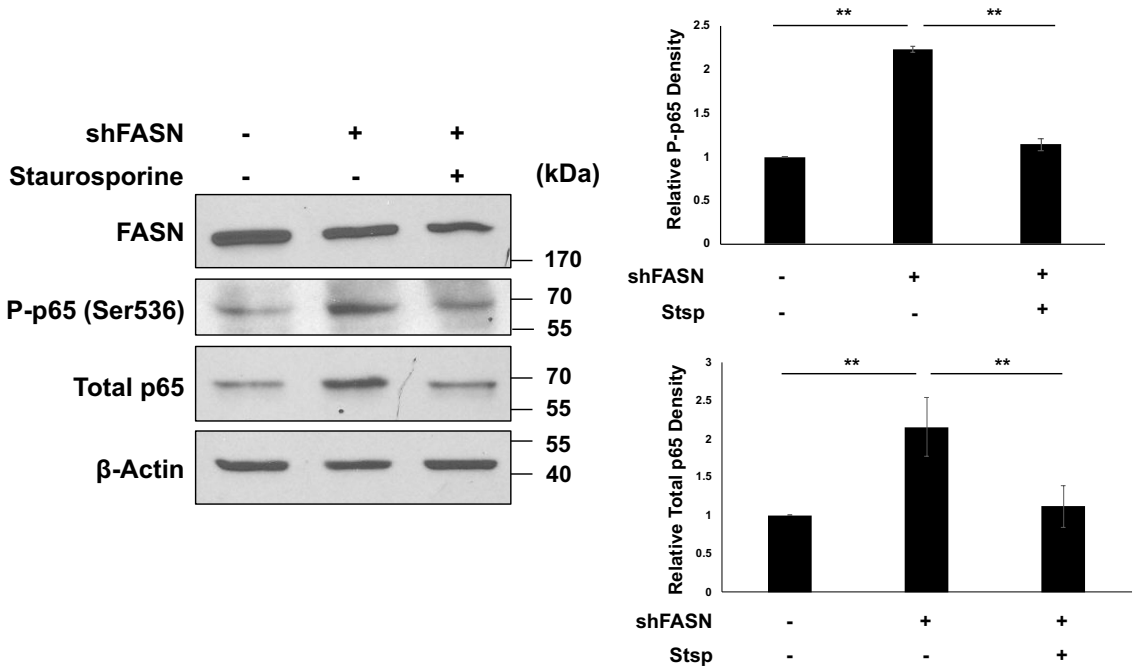


Figure 17. Kinase inhibition reduces p65 protein level.

Western blot analysis of M3K/shFASN cells treated with 100 nM staurosporine or vehicle (DMSO) for 2 hours followed by stimulation with 50 μg/mL TNF-α for 1 hour prior to harvesting cells for Western blot to activate the NF-κB signaling pathway. X-ray film images were quantified using ImageJ. Potential differences in p65 level were determined using one-way ANOVA with Tukey post hoc test. **p<0.01. Bar graphs are a representation of three independent experiments. Error bars represent standard deviation from the mean.

3.14. FASN expression negatively regulates p65 Ser536 phosphorylation

Due to the apparent link between FASN, kinase activity, and p65 protein stability, I next attempted to link FASN with specific p65 phosphorylation sites. As stated earlier in this thesis, phosphorylation of the p65 subunit of NF-κB has long been characterized as being correlated in many cases with NF-κB activation, and the phosphorylation site most commonly associated with NF-κB activation is Ser536 (Chen et al. 2005). Further, it has been found that phosphorylation of Ser536 of p65 takes place following Ser32 and Ser36 phosphorylation of IκBα and alters the timing of translocation of p65 to the nucleus, thereby suggesting this phosphorylation event may affect p65 interaction with IκBα and potentially its trafficking and stability (Mattioli et al. 2004).

As a result of this established relationship and the apparent link between FASN and this phosphorylation site determined with staurosporine treatment, the potential effects of FASN expression changes on p65 phosphorylation at Ser536 were investigated. As shown in **Figure 18**, when FASN was knocked down in M3K cells, this led to an increase in p65 Ser536 phosphorylation relative to scrambled shRNA control cells when the NF-κB pathway was stimulated using TNF-α treatment, whereas, when FASN was overexpressed in MDA-MB-436 cells, this led to a corresponding decrease in p65 Ser536 phosphorylation relative to vector control cells in the presence of TNF-α stimulation. From this data, FASN appears to negatively regulate the phosphorylation of p65 at Ser536.

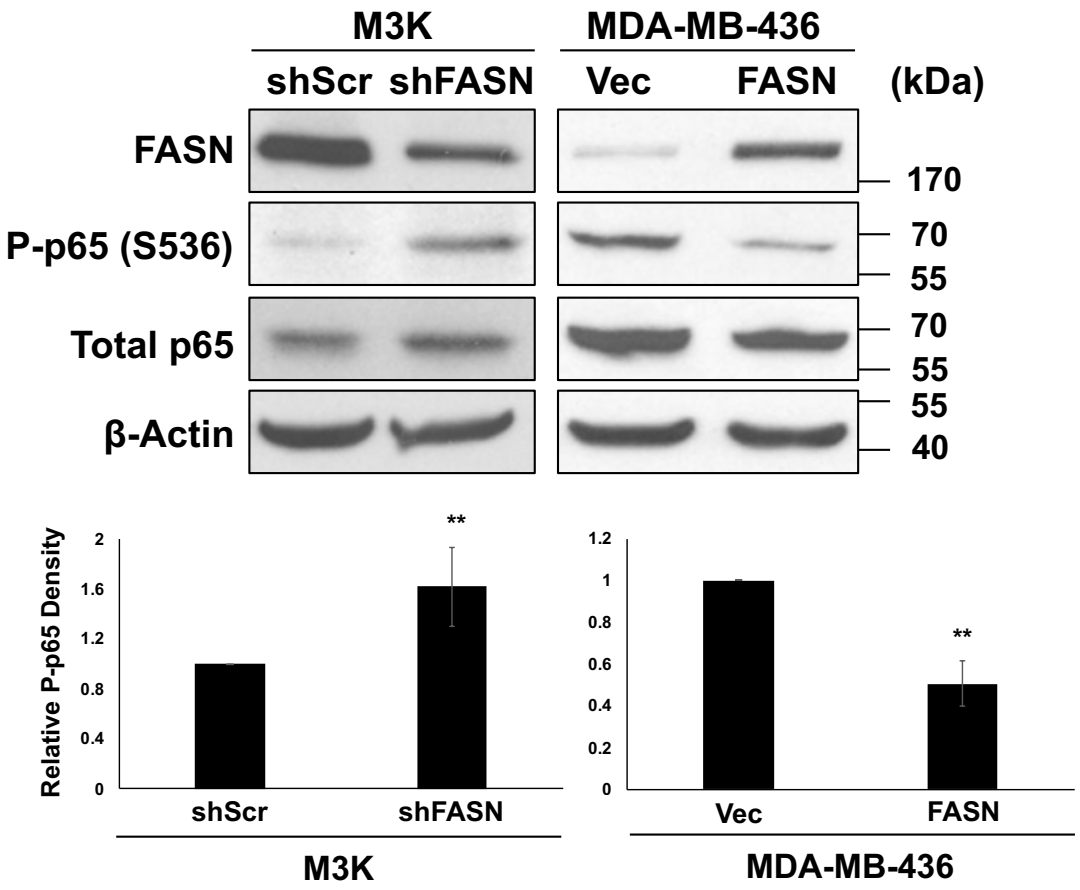


Figure 18. FASN expression negatively regulates p65 Ser536 phosphorylation. Western blot analysis using M3K/shFASN cells along with M3K/shScr control cells and MDA-MB-436/FASN cells along with MDA-MB-436/Vec control cells for phosphorylation of p65 at Ser536. Cells were stimulated with 50 μg/mL TNF-α for 1 hour prior to harvesting cells for Western blot to activate the NF-κB signaling pathway. X-ray film images were quantified using ImageJ. Potential differences between groups were analyzed using Student's *t*-test. **p<0.01. Bar graphs represent three independent experiments. Error bars denote standard deviation from the mean.

3.15. FASN activity negatively regulates p65 Ser536 phosphorylation

I next sought to investigate the relationship between FASN activity and p65 Ser536 phosphorylation. To do so, breast cancer cell lines M3K, MDA-MB-468, and MDA-MB-231 were pre-treated with increasing concentrations of the FASN inhibitor cerulenin for a period of 72 hours, then stimulated with 50 μg/mL TNF-α for 1 hour before cell lysate preparation and Western blot analysis of Ser536-phosphorylated p65 and total p65. As shown in **Figure 19**, increasing concentrations of cerulenin resulted in a dose-dependent increase in phosphorylation of p65 at Ser536, as well as a dose-dependent increase in total p65 as seen earlier in all three breast cancer cell lines tested. From this data, I can conclude that, similar to FASN expression, FASN activity negatively regulates p65 phosphorylation at Ser536, thereby suggesting that Ser536 could potentially be involved in the regulation of total p65 level.

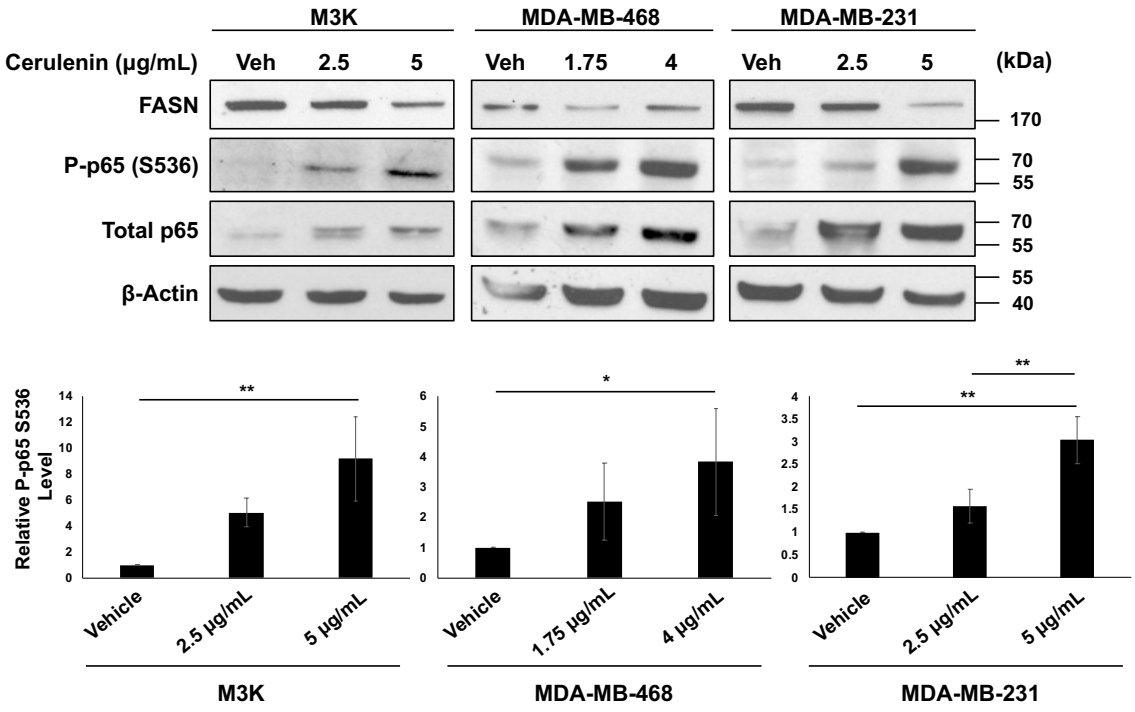


Figure 19. FASN activity negatively regulates p65 Ser536 phosphorylation. Western blot analysis of M3K, MDA-MB-468, and MDA-MB-231 cells pre-treated for 72 hours with increasing concentrations of cerulenin, followed by stimulation with 50 μg/mL TNF-α for 1 hour. Blots were probed for P-p65 Ser536 and total p65. X-ray film images were quantified using ImageJ. Potential significant differences between groups were determined using one-way ANOVA with Tukey post hoc test. *$p<0.05$; **$p<0.01$. Bar graphs for quantification are representative of three independent experiments. Error bars represent standard deviation from the mean.

3.16. p65 Ser536 phosphorylation does not appear to be involved in regulating p65 protein stability

Based on the above observations, I hypothesized that phosphorylation of Ser536 may play a role in regulating p65 protein stability. In order to test this hypothesis, I utilized a kinase inhibitor, BMS-345541, specific to IKK, as IKKα and IKKβ are the primary kinases known to be responsible for phosphorylation of p65 at Ser536 as described earlier (Buss et al. 2004; Lawrence et al. 2005; Sakurai et al. 1999; Sizemore et al. 2002). This inhibitor has been found to inhibit IKKβ-mediated phosphorylation of IκBα with high potency, exhibiting an IC_{50} of 0.3 μM against IKKβ (Burke et al. 2003). Although BMS-345541 exhibits somewhat lower potency towards IKKα, BMS-345541 has also been found to inhibit this isoform significantly with an IC_{50} of 4 μM. It is also believed this inhibitor has substantial selectivity towards IKKβ and IKKα in that the drug did not inhibit other kinases at concentrations up to 100 μM (Burke et al. 2003).

Based on the experimentally-determined IC_{50} values of BMS-345541 in inhibiting both IKK isoforms, M3K/shFASN cells were treated with 5 μM BMS-345541 or vehicle for a period of 2 hours, followed by 1 hour treatment with 50 μg/mL TNF-α to stimulate activation of the NF-κB pathway, wherein cell lysates were prepared, and Western blot analysis was performed for Ser536-phosphorylated p65 and total p65. As shown in **Figure 20**, FASN knockdown in M3K cells led to increases in both phosphorylation of p65 at Ser536 and total p65 level. However, when IKK was inhibited with BMS-345541, increases in phosphorylation of p65 that were seen with FASN knockdown were ablated, as

was IκBα phosphorylation, but there was no effect on the increases in total p65 that were seen following FASN knockdown. This result indicates that, despite the ability of FASN expression changes to affect Ser536 phosphorylation of p65, the effects of FASN expression on p65 Ser536 phosphorylation are not responsible for the effects of FASN on total p65 protein level.

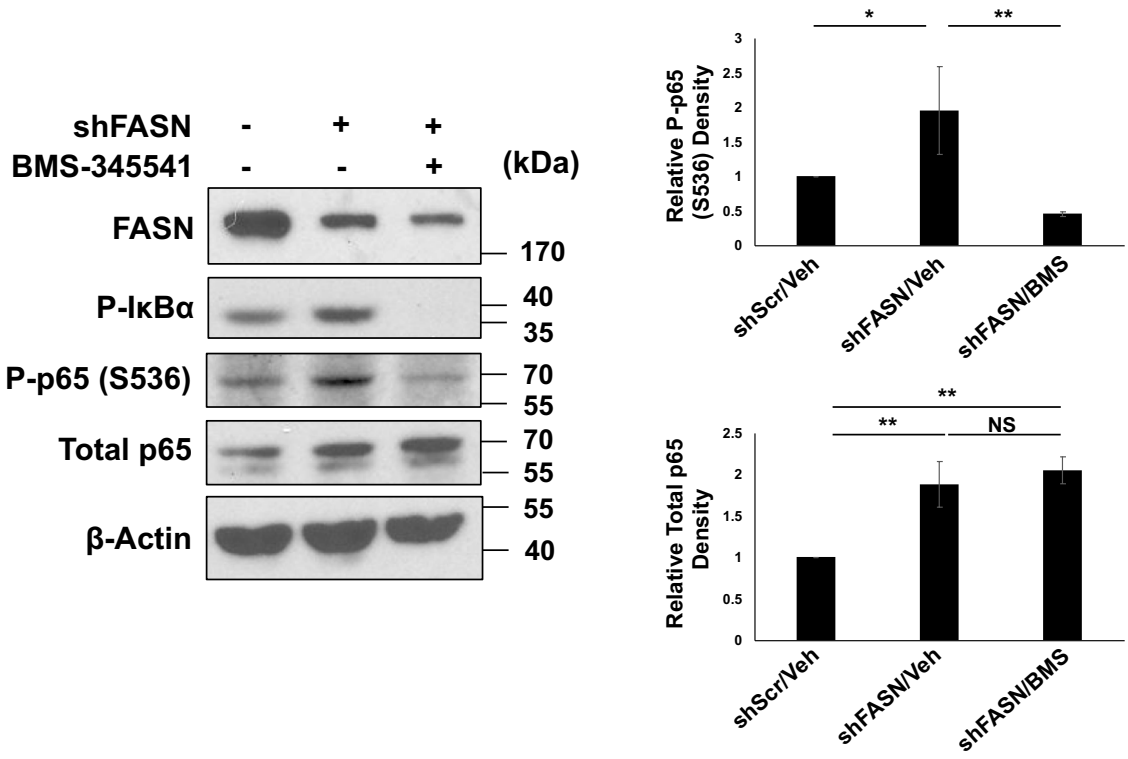


Figure 20. p65 Ser536 phosphorylation does not appear to be involved in regulating p65 protein stability.

Western blot analysis of M3K/shFASN cells treated with or without 5 μM IKK inhibitor BMS-345541 or vehicle (DMSO) for 2 hours followed by 50 μg/mL TNF-α for an additional hour. Western blots were probed for phospho-IκBα, P-p65 (Ser536), and total p65. Images were quantified using ImageJ. Differences between treatment groups were determined using one-way ANOVA with Tukey post hoc test. *$p<0.05$; **$p<0.01$. Bar graphs are representative of three independent experiments. Error bars represent standard deviation from the mean.

3.17. FASN regulation of p65 protein stability involves Thr254 of p65

Another p65 phosphorylation site that has been linked with NF-κB activation is Thr254. Further, phosphorylation of p65 at Thr254 has also been directly linked to increased p65 protein stability (Ryo et al. 2003). I observed that, when Thr254 was mutated in mouse embryonic fibroblast (MEF) cells, p65 protein stability was substantially reduced relative to WT p65. Also, when the proteasome inhibitor MG132 was introduced, this resulted in a rescue in total p65 level in the presence of the Thr254 mutation.

As a result, I hypothesized that FASN may impact p65 protein stability by modulating the phosphorylation of p65 at Thr254 or otherwise by modulating proteins that interact with this phosphorylation site. In order to test the potential involvement of Thr254 in FASN regulation of p65, I initially performed site-directed mutagenesis to mutate the Thr254 site to Ala. This was performed using a pcDNA(3.1)-WT-p65-FLAG plasmid generously provided by Dr. Tao Lu as a template.

Following generation of the T254A mutant plasmid, I next transfected both M3K/shFASN cells and M3K/shScr control cells with either empty pcDNA(3.1) vector as a negative control, pcDNA(3.1)-WT-p65-FLAG, or pcDNA(3.1)-p65-T254A-FLAG for 24 hours, followed by a time course treatment using cycloheximide, which halts global protein synthesis in the cell, at 60 μg/mL for 0 to 12 hours, harvesting cells every 4 hours. After harvesting cells at the indicated time points, Western blot analysis was performed using a FLAG tag antibody in

order to determine the effects of cycloheximide treatment on ectopic p65 expression in the cell over time.

As shown in **Figure 21A**, protein half-life of p65 in M3K/shFASN cells transfected with WT p65 is increased compared to M3K/shScr control cells transfected with WT p65, indicating, similar to endogenous p65, that high levels of FASN expression can reduce p65 protein stability. However, mutation of Thr254 to Ala in M3K/shFASN cells leads to a disruption in p65 protein stability compared to cells expressing WT p65 (**Figure 21B**). This finding suggests that phosphorylation of p65 at Thr254 is important for maintaining p65 protein stability in breast cancer cells.

Also, mutation of Thr254 to Ala in M3K/shScr control cells results in a highly disrupted half-life of p65-FLAG, further supporting the conclusion that the Thr254 site is involved in the ability of FASN to negatively regulate p65 protein stability (**Figure 21B**). In **Figure 21C**, the relative protein level of total p65 present in each condition over the 12 hour time period is shown graphically using one-phase exponential decay. As shown in the graph, only M3K/shFASN cells transfected with WT-p65-FLAG exhibit stable total p65 protein levels over this time course compared to cells with mutated p65-FLAG at Thr254 and M3K/shScr control cells transfected with WT-p65-FLAG. Taken together, these data suggest that high levels of FASN expression negatively regulate p65 protein stability, wherein, when FASN level is high, this may trigger a signaling mechanism responsible for disrupting phosphorylation of p65 at Thr254 and resulting in decreased p65 protein stability.

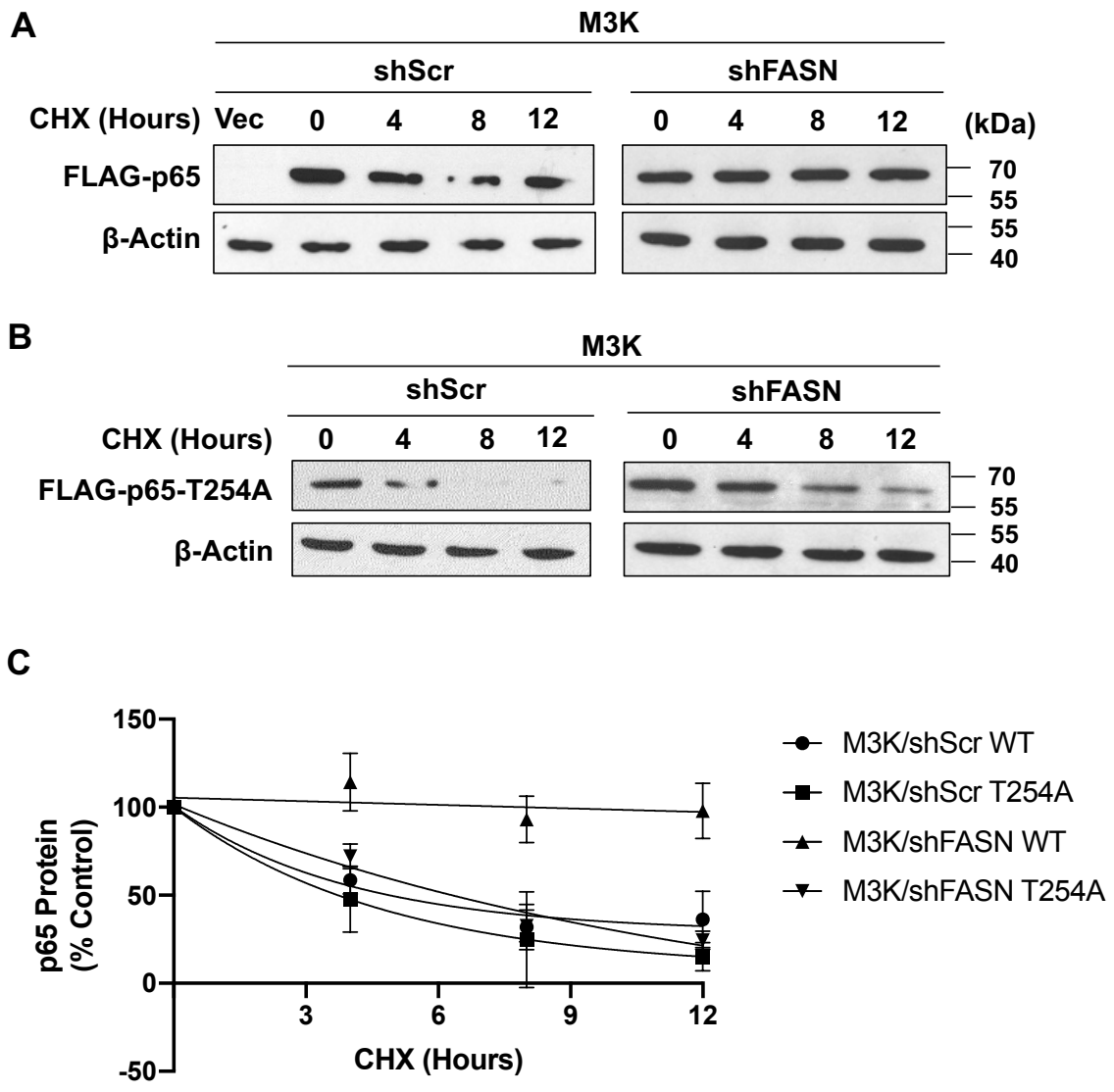


Figure 21. FASN regulation of p65 protein stability involves Thr254 of p65. (A and B) Western blot analysis of M3K/shFASN cells and M3K/shScr control cells transfected with (A) wild type p65-FLAG or (B) p65-FLAG with T254A mutation, treated with 60 μg/mL cycloheximide for 0 to 12 hours, and harvested at the indicated time points before blotting and probing for FLAG tag recognizing tagged p65. (C) Graphical analysis performed in Graph Pad Prism indicating the

percent loss in p65-FLAG protein over time in the presence of cycloheximide in all cell lines tested. Western blots were quantified using ImageJ. Graph was generated using Graph Pad Prism one-phase exponential decay and is representative of three independent experiments. Error bars represent standard deviation from the mean.

3.18. Pin1 knockdown in breast cancer cells decreases total p65 expression

It has been reported that the peptidyl-prolyl *cis/trans* isomerase Pin1 plays a significant role in mediating the enhancement in p65 protein stability that is seen in the presence of p65 Thr254 phosphorylation (Ryo et al. 2003). Specifically, when p65 is present in the nucleus following its activation and is not bound by the inhibitory subunit IκBα, the Thr254 site of p65 is exposed, allowing for its phosphorylation (Ryo et al. 2003). This then creates a binding site for Pin1, which subsequently binds to the Thr254-Pro motif and likely carries out its isomerization activity on p65. The loss of Pin1 was shown to disrupt p65 nuclear accumulation and significantly reduce p65 stability (Ryo et al. 2003).

In order to investigate the potential role for Pin1 in the relationship between p65 Thr254 and p65 protein stability in breast cancer cells, siRNA targeting Pin1 to knock down Pin1 expression was utilized. Western blot analysis utilizing Pin1 siRNA knockdown in M3K/shFASN cells showed that decreasing Pin1 expression results in a reduction in total p65 protein level relative to M3K/shFASN cells transfected with scrambled control siRNA (**Figure 22**). This finding suggests that Pin1 assists in mediating the increases in p65 protein stability that are seen when FASN expression is lost.

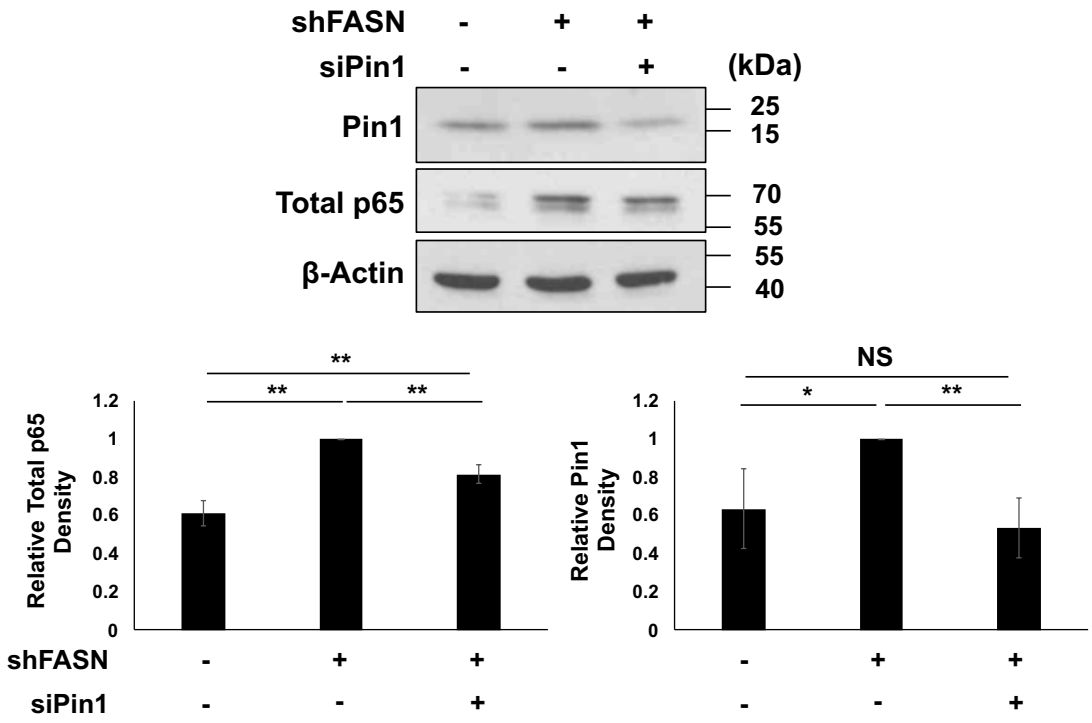


Figure 22. Pin1 knockdown in breast cancer cells decreases total p65 expression.

Western blot analysis of M3K/shFASN cells transfected with siRNA targeting Pin1 or scrambled control siRNA for 48 hours blotting for total p65 and Pin1 to verify knockdown. Images were quantified using ImageJ. Potential significant differences between groups were determined using one-way ANOVA with Tukey post hoc test. *$p<0.05$; **$p<0.01$. Bar graphs are representative of three independent experiments. Error bars represent standard deviation from the mean.

3.19. Pharmacological inhibition of Pin1 in breast cancer cells reduces total p65 expression

Ryo et al. showed that cells that did not express Pin1 exhibited decreased p65 protein stability (Ryo et al. 2003). This finding suggested that the prolyl isomerase activity of Pin1 is responsible, in part, for maintaining the stability of p65, and that this coincides with phosphorylation of p65 at Thr254. As a result, I sought to investigate the potential role of Pin1 activity in regulating total p65 protein level. In order to investigate this potential role, I utilized dipentamethylene thiuram monosulfide (DTM), which has been characterized as a competitive inhibitor that specifically inhibits the pepidyl-prolyl *cis/trans* isomerase activity of Pin1 (Tatara et al. 2009).

Specifically, M3K/shFASN cells were treated with 10 μM DTM or vehicle (DMSO) for a period of 48 hours before performing Western blot analysis to determine the effects of Pin1 inhibition on total p65 level. As shown in **Figure 23**, inhibition of Pin1 with DTM resulted in a significant decrease in total p65 level in M3K/shFASN cells relative to control treatment. In fact, treatment with DTM completely ablated the increases in total p65 level that are seen with FASN knockdown and returned p65 level to a level similar to that of vehicle-treated M3K/shScr control cells. This finding, along with the effects of Pin1 siRNA on p65, indicates that the isomerase activity of Pin1 plays a significant role in mediating p65 protein stability in the absence of FASN.

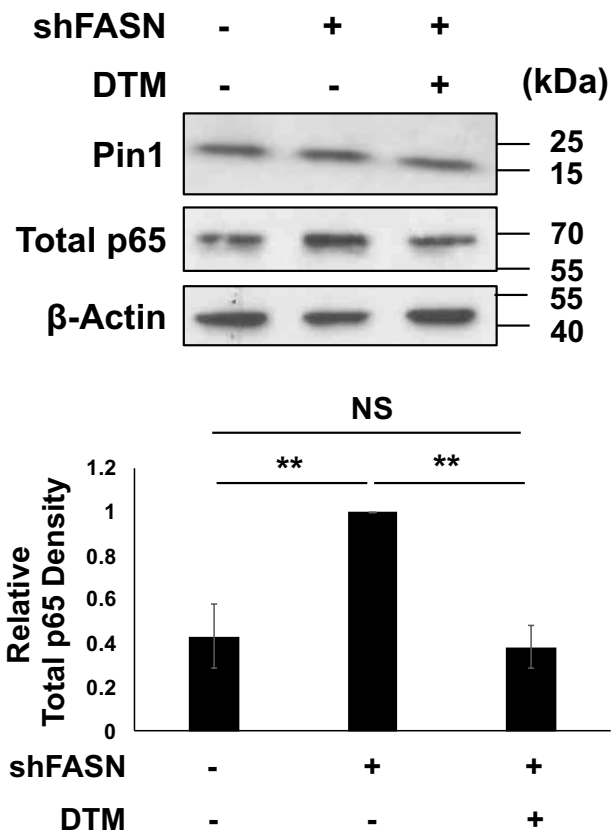


Figure 23. Pin1 pharmacological inhibition in breast cancer cells reduces total p65 expression.

M3K/shFASN cells treated with 10 μM DTM or vehicle (DMSO) for 48 hours followed by Western blot analysis for total p65 and compared to M3K/shScr control cells treated with vehicle (DMSO). Images were quantified using ImageJ. Potential differences between groups were calculated using one-way ANOVA with Tukey post hoc test. **$p<0.01$. Bar graphs are representative of three independent experiments. Error bars indicate standard deviation from the mean.

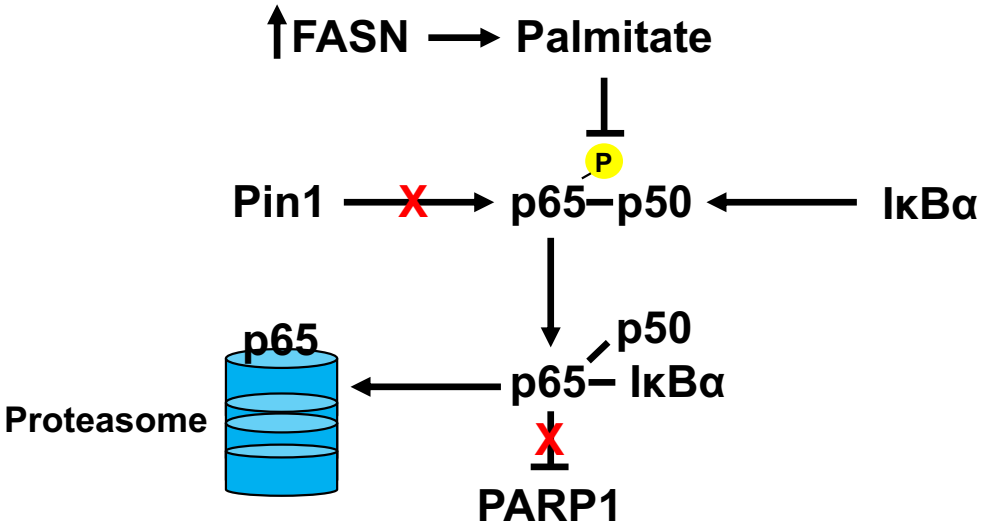
3.20. Proposed mechanism of FASN regulation of NF-κB/p65

In summary, when FASN expression is high in breast cancer cells, FASN activity results in increased production of the saturated fatty acid palmitate as the primary product. Palmitate then plays a role in an unknown signaling cascade that culminates in either preventing the phosphorylation of p65 at Thr254 or, otherwise, disrupting the phosphorylation of p65 at Thr254. As a result of the lack of phosphorylated p65, Pin1 is unable to bind p65 at its Thr254-Pro motif. Due to the lack of Pin1 binding, p65 is not isomerized and does not undergo a conformational change. Consequently, p65 likely is bound by the inhibitory subunit IκBα, as was demonstrated in the absence of Pin1 binding by Ryo et al. As a result, continuous activation of the NF-κB signaling pathway does not occur, and p65 is targeted for poly-ubiquitination and proteasomal degradation. Ultimately, in the overall mechanism of FASN regulation of NHEJ DNA repair, this process subsequently has the effect of relieving the ability of NF-κB to inhibit PARP1, thereby resulting in an increase in PARP1 expression. This mechanism is shown graphically in **Figure 24A**.

Contrarily, when FASN expression is low in breast cancer cells, or when FASN is inhibited pharmacologically, FASN activity is disrupted, resulting in decreased production of its catalytic product palmitate. As a consequence of the loss of FASN and palmitate, p65 is likely able to be phosphorylated at Thr254. This phosphorylation event signals for the binding of Pin1, and Pin1 subsequently carries out its peptidyl-prolyl *cis/trans* isomerase activity on p65, resulting in p65 undergoing a conformational change. This conformational

change disrupts the ability of p65 to bind with IκBα but does not affect its interaction with its heterodimeric partner p50 (Ryo et al. 2003). As a result, the p65/p50 heterodimer is able to remain in the nucleus, resulting in sustained NF-κB activation and the transcription of specific target genes such as TNF-α. This mechanism is shown graphically in **Figure 24B**.

A


FASN
Palmitate
P
Pin1
p65–p50
IκBα
p65
p50
p65– IκBα
Proteasome
PARP1

B

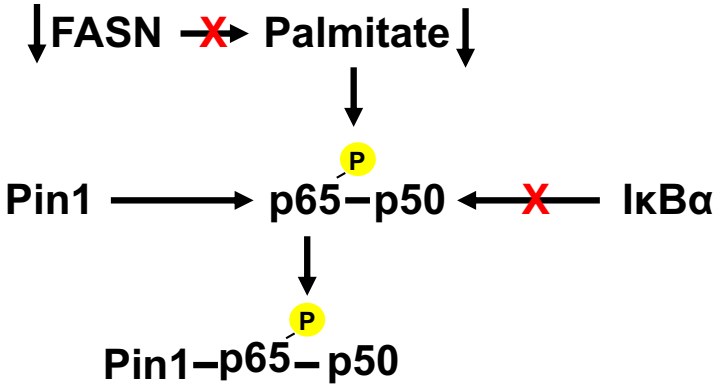

FASN
Palmitate
P
Pin1
p65–p50
IκBα
P
Pin1–p65– p50

Figure 24. Proposed mechanism of FASN regulation of NF-κB/p65.

(A) When FASN expression is high in breast cancer cells, palmitate is produced as the end product of FASN activity. An unknown signaling cascade then leads to either the dephosphorylation of p65 at Th254 or leads to the inhibition of kinase activity that prevents phosphorylation of p65 at Thr254. As a result, Pin1 does not recognize and bind p65, and p65 does not undergo a conformational change. Instead, p65 is likely bound by the NF-κB inhibitory subunit IκBα and exported from the nucleus to be degraded through the proteasome. Loss of p65 prevents sustained NF-κB activation and, subsequently, allows for an upregulation of PARP1. (B) When FASN expression is low or inhibited pharmacologically in breast cancer cells, palmitate production and free palmitate present in the cell is reduced. Reduced palmitate level either disrupts a signaling cascade that results in dephosphorylation of p65 at Thr254 or rather allows for increased activity of an unknown kinase that acts to phosphorylate p65 at Thr254. Presence of p65 in its phosphorylated form then allows for the binding of Pin1, which binds the phospho-Thr254-Pro motif of p65 and induces a conformational change in the p65 protein structure, which serves to confer increased p65 protein stability and disrupts the potential interaction between p65 and IκBα. This increased p65 protein stability leads to increased p65 nuclear residence and accumulation, as well as increased NF-κB activity.

Chapter 4: Discussion

4.1. Summary of Findings

Previous research has uncovered a mechanism wherein FASN contributes to increased cancer cell survival through the upregulation of NHEJ DNA repair activity involving the inhibition of NF-κB (Wu et al. 2016). This thesis explores the specific pathway responsible for FASN regulation of NF-κB in breast cancer cells with the goal of uncovering in detail the cell signaling relationship exhibited by these two genes. As summarized schematically in **Figure 24**, the results shown in this thesis suggest that, when FASN expression is high in breast cancer cells, as is often the case in clinical cases of breast cancer, the high level of FASN expression and corresponding high amounts of palmitate are able to negatively regulate expression of total p65 at the protein level, while not affecting p65 at the level of transcription. It was also found that pharmacological inhibition of FASN with cerulenin produces the same effect on total p65. This negative regulation of NF-κB/p65 also was determined to correspond to negative regulation of NF-κB activation involving a reduction in NF-κB transcriptional activity and a disruption in the ability of NF-κB to regulate gene expression of TNF-α and PARP1. In addition, it was determined that high levels of FASN expression result in a reduction in p65 protein stability, and that the increased protein turnover of p65 with high FASN expression resulted from increased p65 poly-ubiquitination and proteasomal degradation. Further, I observed that the regulation of p65 protein stability involves the phosphorylation site Thr254, in that

mutation of this residue disrupts p65 protein stability, even in the absence of high levels of FASN expression. A lack of phosphorylation of this site also likely affects p65 stability specifically by preventing the interaction of p65 with the peptidyl-prolyl *cis/trans* isomerase Pin1, as knockdown and pharmacological inhibition of Pin1 resulted in a decrease in p65 protein level.

In the bigger picture of FASN signaling in breast cancer cells that Dr. Zhang and his colleagues have been unraveling over the last several years of research, this process prevents the ability of NF-κB to inhibit PARP1, which has been shown to be specifically involved in up-regulating mechanisms of DNA repair through NHEJ activity to confer increased cancer cell survival (Wu et al. 2016).

4.2. Impact of FASN Inhibition on FASN Expression

Cerulenin is a long-studied small molecule inhibitor of the KS domain of FASN. In this thesis, I observed that inhibition of FASN with cerulenin not only produced cellular effects on NF-κB/p65, but also led to a corresponding decrease in FASN expression, as indicated by reduced FASN protein level by Western blot. Many studies have shown similar depletions in FASN expression with cerulenin treatment (Li et al. 2014; Bauerschlag et al. 2015; Lee et al. 2017; Tian et al. 2018). Many potential mechanisms could be responsible for this reduction in expression that occurs with FASN inhibition. One potential explanation is that the binding of cerulenin to the KS domain of FASN could induce a conformational

change in the FASN protein structure that disrupts the protein stability of FASN to induce increased FASN degradation.

Research has shown that FASN expression can be sustained through FASN deubiquitination by the isopeptidase ubiquitin-specific protease-2a (USP2a), which prevents FASN proteasomal degradation and was first determined in prostate cancer cells (Graner et al. 2004). This finding suggests the possibility that FASN inhibition with cerulenin or other FASN inhibitors may prevent the ability of ubiquitination marks to be removed from FASN, potentially due to reduced access of deubiquitinating enzymes to FASN in the presence of inhibitor binding or through another mechanism.

Another study also found that acetylation of FASN by histone acetyltransferase 8 (KAT8) promoted the association of FASN with an E3 ubiquitin ligase to result in FASN proteasomal degradation, whereas the removal of acetylation marks catalyzed by histone deacetylase 3 (HDAC3) resulted in increased FASN expression (Lin et al. 2016). This suggests that FASN inhibition has the potential to either promote the acetylation of FASN or to reduce access of HDAC3 or other deacetylating enzymes to FASN proteins to result in the promotion of FASN degradation. The possibility that cerulenin or other inhibitors targeted against FASN induce FASN degradation through these mechanisms could be investigated by utilizing proteasome inhibitors in the presence of FASN inhibition.

Interestingly, a study in ovarian cancer cells has shown that, while cerulenin treatment reduced FASN expression at the protein level,

supplementation of cells with palmitate in combination with cerulenin treatment appeared to result in a rescue in FASN protein level (Bauerschlag et al. 2015). This may indicate that free fatty acid levels in the cell may impact the expression level of FASN in the presence of FASN inhibition. Further, it is widely believed that the accumulation of malonyl-CoA that results from FASN inhibition with cerulenin and other inhibitors is a main cause of cancer cell apoptosis (Menendez and Lupu 2007). As a result, it is possible that the accumulation of malonyl-CoA and other fatty acid biosynthesis intermediates play a role in the destabilization of FASN in the presence of FASN inhibition.

4.3. Potential Causes for FASN and Palmitate Effects on p65 Phosphorylation

The data presented in this thesis have shown that FASN affects the stability of p65 protein, and that the specific phosphorylation site Thr254 is involved in p65 protein stability, thereby indicating that FASN may affect phosphorylation of p65 at this site. Though it appears palmitate is likely involved in regulating p65 protein stability downstream of FASN according to the data in **Figure 8** of this thesis, it remains largely unclear how FASN is able to impact phosphorylation of p65 at Thr254 to affect p65 protein stability. As discussed earlier in the introduction, palmitate is known to play a significant role in cancers through a variety of processes. Specifically, it has been shown that protein modification through palmitoylation of cysteine residues can have a direct effect on the activity of signaling molecules such as Wnt proteins and Ras GTPases

(Rohrig and Schulze 2016; Eisenberg et al. 2013). As a result, a logical hypothesis for the role of FASN in affecting phosphorylation of p65 at Thr254 would likely begin with investigation into a potential role for protein palmitoylation, whether that be directly on p65 to perhaps affect the conformation of the protein and disrupt phosphorylation, or on a different protein to induce a subsequent effect on p65 phosphorylation.

As shown in **Appendix A**, blocking palmitoylation with an inhibitor resulted in a decrease in total p65 protein level in M3K doxorubicin resistant breast cancer cells. This finding suggests that high FASN expression does not lead to direct palmitoylation of p65 to suppress p65 expression. Further supporting this finding, many proteomics studies have been carried out to determine palmitoylated proteins in various mammalian genomes. Compilation studies of these protein palmitoylomes, though finding many proteins appearing to be palmitoylated that have significant roles in cancer, did not find p65 or any other NF-κB family proteins to be palmitoylated (Sanders et al. 2015; Ko and Dixon 2018).

Though it does not appear likely FASN leads to the palmitoylation of p65, it is possible FASN impacts p65 expression through an indirect mechanism that involves palmitoylation, seeing as blocking palmitoylation activity resulted in a decrease in p65 protein level. In such a mechanism, when FASN expression is high, FASN production of palmitate could lead to the palmitoylation of a kinase, subsequently leading to the suppression of the activity of that kinase to prevent phosphorylation of p65 at Thr254 and disrupt p65 protein stability. Another

possible mechanism could involve FASN-mediated palmitoylation of a phosphatase, wherein palmitoylation results in increased phosphatase activity.

Neither the canonical kinase responsible for the phosphorylation of p65 at Thr254, nor any phosphatases responsible for removing phosphoryl groups from this site are known. Based upon the uncovered relationship between FASN and this p65 phosphorylation site, a logical place to start investigating a potential kinase or phosphatase in the context of breast cancer cells would be to first investigate those known to have a mechanistic relationship with FASN.

One particular kinase, PKC, has been linked to both FASN and p65. Specifically, one study has shown that, in response to FASN inhibition with the cerulenin derivative C93, the classical PKC isoform PKCα was activated and able to phosphorylate IκBα to result in its degradation and the subsequent activation of NF-κB (Lemmon et al. 2011). Further, multiple PKC isoforms have been found to be pamitoylated in proteomics studies, while the palmitoylation of PKCε has been verified in *in vitro* studies (Sanders et al. 2015; Dasgupta et al. 2011). Interestingly, PKC is commonly known to translocate to the nucleus upon its activation. Given that it is believed the phosphorylated form of p65 is likely present in the nucleus, this suggests that activated PKC and p65 in this context would both be localized in the nucleus. As a result, it is possible FASN may affect the palmitoylation of a specific PKC isoform, thereby affecting its ability to phosphorylate p65 at Thr254.

Multiple studies have also linked FASN inhibition to the activation of AMP-activated protein kinase (AMPK) (Landree et al. 2004; Zhou et al. 2007). Though

it has not been found in palmitoylome studies that AMPK can be palmitoylated, it is possible AMPK is involved in this process, as it has been shown that AMPK activation can increase NF-κB nuclear translocation and DNA binding activity (Liu et al. 2010). As a result, examining the potential relationship between FASN, NF-κB/p65, and these kinases may lead to the determination of a more detailed mechanistic understanding of the ability of FASN to regulate NF-κB/p65.

In further consideration of the effects on total p65 level with the inhibition of palmitoylation activity shown in **Appendix A**, though 2-bromopalmitate is known to block palmitoylation of proteins, there is also evidence to suggest that it can behave similarly to palmitate in cells with respect to signaling processes (Resh 2006). For example, it has been shown that 2-bromopalmitate treatment can lead to gene expression changes through activation of peroxisome proliferator-activated receptors (PPARs), which are receptors for which palmitate is a common ligand (Brandes et al. 1995; Bastie et al. 2000). As a result, it must be considered that 2-bromopalmitate treatment in breast cancer cells could, in fact, be acting as a palmitate analog and could be affecting total p65 protein level not through a mechanism involving inhibition of palmitoylation, but rather through a cell signaling mechanism that is independent of palmitoylation activity and mirrors the effects of exogenous palmitate treatment. If this is the case, it is possible palmitate affects total p65 protein stability by a mechanism involving its other roles in cell signaling, such as modulation of membrane composition and lipid raft formation.

4.4. Relationship Between FASN and Kinase Activity

Due to previous research showing a relationship between phosphorylation of p65 and protein stability, I hypothesized that the effect of FASN on p65 protein stability may be the result of an effect exerted by FASN on phosphorylation. To initially investigate this hypothesis, a general kinase inhibitor known as staurosporine was utilized to determine if kinase activity may play a role in the effects of FASN on p65, and it was determined that staurosporine ablated the increases in total p65 protein level that are seen with FASN knockdown (**Figure 17**).

While this initial experiment and conclusion provided insight for the direction of this thesis moving forward and was a logical starting point to investigate the relationship between FASN and p65 phosphorylation, the use of staurosporine as a general kinase inhibitor has caveats that must be explored. The primary mechanism of action of staurosporine is to bind to the ATP binding pocket of target kinases, thereby preventing the binding of ATP and disrupting kinase activity. However, staurosporine binds to kinases with very little selectivity as a consequence of the conserved nature of ATP binding pockets across the broad array of protein kinases. As a result, it may be difficult to determine whether the effects of staurosporine treatment on total p65 level speak to a causal relationship between FASN, activity of a specific kinase, and NF-κB/p65, or rather if the effects staurosporine exerts on total p65 level are more indirect, in that relatively global kinase inhibition could lead to effects on potential upstream kinases that may be responsible for regulating the cellular mechanisms and

molecular players within the signaling relationship that exists between FASN and NF-κB/p65.

Potential evidence for signaling consequences of inhibiting kinase activity on a relatively global level exist in that staurosporine is known to induce apoptosis in a variety of cell types. While many potential signaling mechanisms have been suggested for the role of staurosporine in triggering apoptosis, one mechanism commonly found is the activation of caspases, specifically caspase-3 and caspase-9 (Chae et al. 2000; Malsy et al. 2019; Belmokhtar et al. 2001). Interestingly, one of these studies also found that staurosporine treatment actually led to an increase in NF-κB transcriptional activity (Chae et al. 2000). As staurosporine treatment resulted in a decrease in total p65 protein level, this suggests that the global kinase inhibition of staurosporine treatment may impact NF-κB signaling through a variety of different mechanisms.

4.5. Relationship Between FASN, NF-κB, and TNF-α

The data in **Figure 12** of this thesis has shown that FASN negatively regulates the expression of TNF-α, a canonical target gene of NF-κB signaling activation (Falvo et al. 2010; Liu et al. 2000). This finding was also consistent with previous research in the Zhang lab, which showed that FASN expression was negatively correlated with TNF-α expression, as well as TNF-α promoter activity (Liu et al. 2013).

While TNF-α is one of the genes commonly regulated by NF-κB, TNF-α is also one of the more common stimuli known to activate the NF-κB signaling

pathway, and, as a result, regulation of the expression of these two genes is often heavily intertwined (Aggarwal 2003). As a result, it would seem possible or even likely that, in a situation of reduced FASN expression or the presence of FASN inhibition, increased expression of NF-κB/p65 and increased NF-κB activity would promote increased expression and secretion of TNF-α, which could have the potential to create a positive feedback loop between TNF-α and NF-κB.

Literature evidence exists that would support the likelihood of this possibility. For instance, in acute myeloid leukemia (AML), it has been found that constitutive activity of NF-κB was promoted and maintained through continued secretion of TNF-α leading to a feedback loop of continuous autocrine signaling activation of NF-κB (Kagoya et al. 2012; Kagoya et al. 2014). This feedback loop activation has also been linked to other pathologies including rheumatoid arthritis and inflammatory bowel disease (Papa et al. 2004). As a result, it is worth consideration that increased FASN expression in breast cancer cells may result in decreased p65 protein stability, in part, in response to the disruption of a feedback loop between TNF-α and NF-κB that provides for continuous NF-κB activation and, consequently, increased p65 protein stability through a disruption in p65 proteasomal degradation.

4.6. Relationship Between FASN, NF-κB, and PARP1

Previous research has determined a relationship between FASN, NF-κB, and PARP1, wherein FASN was able to upregulate PARP1 by suppressing the ability of NF-κB to inhibit PARP1 expression (Wu et al. 2016). As shown in

Figure 13, this relationship also holds true in breast cancer cells, wherein PARP1 is positively regulated by FASN expression as opposed to the negative regulation of NF-κB/p65 by FASN. As discussed earlier, previous research uncovered a composite binding site in the PARP1 promoter for p65 and another transcription factor, SP1 (Wu et al. 2016). It was also determined that, while overexpression of p65 suppressed PARP1 expression in pancreatic cancer cells, accompanying overexpression of SP1 in the presence of p65 suppressed the ability of p65 to negatively regulate PARP1. Specifically, when higher amounts of SP1 were transfected into pancreatic cancer cells, SP1 was able to overcome p65 to upregulate PARP1. Interestingly, this phenomenon is not unique to this mechanism as it has been found that NF-κB proteins can antagonize the binding of SP1 proteins to the P-selectin promoter (Hirano et al. 1998).

In the same study, exogenous palmitate treatment was able to upregulate SP1 protein level (Wu et al. 2016). Mechanistically, this finding is in concert with the finding in this thesis in **Figure 8** that exogenous palmitate treatment was able to suppress p65 protein level. As palmitate appears to affect both SP1 and p65 expression, it is possible that the unknown mechanisms through which FASN and palmitate impact SP1 and p65 are to some degree intertwined, as with the composite sites in the PARP1 promoter involved in SP1 and NF-κB regulation of PARP1 expression and activity.

4.7. Relationship Between FASN and Pin1 Expression

In this thesis, I have hypothesized that there is a relationship between NF-κB/p65 and Pin1, wherein Pin1 assists in the stabilization of p65 protein in the absence of FASN expression or when FASN activity is inhibited. Specifically, I determined that both Pin1 knockdown and Pin1 pharmacological inhibition resulted in a decrease in total p65 protein level. In **Figure 22**, there was also found to be a slight increase in Pin1 protein level when FASN expression was knocked down in M3K doxorubicin resistant breast cancer cells. This finding is not wholly unexpected given that it follows the cellular mechanism determined in this thesis, wherein FASN expression is also negatively correlated with total p65 expression.

However, the finding that FASN exhibits a negative relationship with Pin1 expression in breast cancer cells is actually contradictory to a previous study, which determined that FASN exhibited a positive relationship with Pin1, wherein knockdown of Pin1 with siRNA and pharmacological inhibition of Pin1 reduced FASN protein level, and Pin1 pharmacological inhibition sensitized cells to HER2-targeted treatment with trastuzumab (Yun et al. 2014). It is worth noting, however, that this study was conducted solely utilizing HER2-positive breast cancer cell lines (SK-Br-3 and BT-474). This caveat is worth mentioning because previous research suggests the possibility that FASN may play a specific role in breast cancer cells that overexpress the HER2 receptor. To this end, previous research in the Zhang lab found no correlation between FASN expression level and sensitivity of MCF7 breast cancer cells—which express HER2 but at a low

level—to drugs that affect microtubules including paclitaxel and vinblastine (Liu et al. 2013). While other groups have shown that FASN inhibitors can sensitize breast cancer cells to microtubule inhibiting drugs, the ability of FASN inhibitors to sensitize cells was variable depending upon the cell line tested (Menendez et al. 2004; Menendez et al. 2004; Menendez et al. 2005). HER2 positive breast cancer cells, however, were consistently sensitized by multiple FASN inhibitors to multiple drugs targeted to microtubules in these studies. This suggests there may be some specific mechanism or interaction between FASN and HER2 in HER2-overexpressing breast cancer cells that allows for inhibitors targeted to these two proteins to interact synergistically to kill cancer cells. Therefore, this potentially unique relationship between FASN and HER2 in HER2-overexpressing breast cancer cells may account for the contradictory relationship between FASN and Pin1 in our study compared with the findings of Yun et al.

4.8. Cellular Consequences of the Relationship Between FASN and NF-κB

The characterized roles of NF-κB in cancer cells are variable in that NF-κB signaling activation, depending on the circumstance or the cell type, can have differing consequences on the cell. As described earlier in the introduction, the canonical role of NF-κB activation is typically oncogenic, characterized by the prevention of apoptosis, promotion of angiogenesis, and promotion of cellular proliferation. However, this phenomenon does not hold true in all settings, as NF-κB signaling has also been found to promote cell death in certain instances (Kothny-Wilkes et al. 1998; Strozyk et al. 2006; Bian et al. 2001; Huang and

Johnson et al. 2000). As a result, it is potentially difficult to speculate on the downstream cellular consequences ensuing from the effects of the relationship between FASN and NF-κB/p65. For reference, Lemmon et al. found that, while FASN inhibition induced nuclear translocation and transcriptional activation of NF-κB in lung and prostate cancer cells, they also found that combined inhibition of FASN and NF-κB signaling resulted in increased cell killing compared to FASN inhibition alone (Lemmon et al. 2011). Further, it is worth noting that this phenomenon held true with the combination of FASN knockdown with siRNA and inhibition of NF-κB signaling, though this combined effect on cancer cell death was less pronounced than with pharmacological inhibition of FASN. The findings of this study suggest that, in this specific case, the activation of NF-κB in response to FASN inhibition or loss of FASN expression is, in fact, cytoprotective for cancer cells as blocking the activation resulted in more effective cell killing.

However, there are dissimilarities in the findings in this thesis relative to the findings of Lemmon et al. Specifically, with respect to the gene expression changes implicated in this mechanism as shown in **Figure 13**, FASN was shown to be a positive regulator of the expression of PARP1 in breast cancer cells, as opposed to the negative regulation of NF-κB/p65 by FASN. Dr. Zhang's previous research has shown that p65 regulates PARP1 in this mechanism by suppressing PARP1 expression, whereas the high level of FASN expression present in breast and pancreatic cancer cells suppresses p65 to allow for the upregulation of PARP1 (Wu et al. 2016). As the functional effect of this suppression of p65 and upregulation of PARP1 was an increase in NHEJ DNA

repair activity and an increase in cancer cell survival to promote drug resistance to DNA-damaging agents, this finding suggests that NF-κB/p65 may be acting to promote cancer cell apoptosis in this mechanism. If this is the case, it would suggest that, rather than acting in its well-characterized role of increasing drug resistance by upregulating the expression of pro-survival genes, NF-κB activation downstream of decreased FASN expression or FASN inhibition may act to promote cell death by inhibiting PARP1 and possibly promoting cell death pathways such as the upregulation of Fas ligand (Kasibhatla et al. 1998). Interestingly, it has also been determined that Pin1 can also function in cell death mechanisms, wherein two studies have shown the ability of Pin1 to upregulate p53-mediated apoptosis (Sorrentino et al. 2013; Follis et al. 2015). In correlation with this role of Pin1, it has also been found that NF-κB activation promoted p53-mediated cell death, whereas inhibition of NF-κB suppressed p53-mediated apoptosis, thereby suggesting a link between Pin1, NF-κB, and the promotion of cell death mechanisms (Ryan et al. 2000). However, further study is required to tease out the specific functional outcome of NF-κB/p65 in breast cancer cells in this mechanism.

4.9. Relationship Between FASN and p65 Ser536 Phosphorylation

When attempting to determine a link between FASN, p65 protein stability, and phosphorylation of p65, I first investigated whether the site Ser536 was involved in the ability of FASN to regulate p65 protein stability. In **Figure 20**, I observed that, while decreased FASN expression led to a corresponding

increase in p65 Ser536 phosphorylation, similar to the increases in total p65 protein level with FASN knockdown, loss of p65 Ser536 phosphorylation resulting from IKK inhibition did not subsequently affect total p65 level.

As a result of this finding, the increases in phosphorylation of p65 at Ser536 that are seen in response to FASN knockdown do not appear to be an upstream mechanism responsible for the effects FASN exerts on p65 protein stability. However, despite the conclusion that Ser536 phosphorylation is not involved in FASN regulation of p65 protein stability, FASN expression changes do appear to exhibit a relationship with Ser536 phosphorylation. It is possible that the increases in p65 Ser536 phosphorylation that are seen with FASN knockdown are merely a correlating effect on the ratio of phosphorylated p65 protein to total p65 protein, wherein as the total protein level increases, the level of Ser536 phosphorylation increases proportionally.

It is worth noting, however, that Ser536 phosphorylation of p65 is heavily linked to NF-κB activation. With this in mind, the increase in Ser536 phosphorylation seen with FASN knockdown aligns with the increase in NF-κB activity that is seen with FASN knockdown (**Figure 11**). In the context of the binding of Pin1 with p65 and the presence of phosphorylation of p65 at Thr254, structural data has indicated that both Thr254 phosphorylation and Pin1 binding occurs following the phosphorylation of IκBα and its subsequent release from p65 (Ryo et al. 2003). More specifically, this study showed that p65 is likely phosphorylated at Thr254 following nuclear translocation and transcriptional activation, as it was shown that Thr254 phosphorylated p65 did not interact with

IκBα and remained accumulated in the nucleus, indicating that this phosphorylation event prevented the ability of IκBα to export p65 from the nucleus and induce its proteasomal degradation (Ryo et al. 2003). On the contrary, it has been found that following stimulation, such as with TNF-α, Ser536 phosphorylation occurs relatively quickly while p65 and IκBα are still in complex in the cytoplasm (Mattioli et al. 2004). Further, it has also been found that only a very small percentage of Ser536 phosphorylated p65 is found in the nucleus, but is rather found predominantly in the perinuclear space, as it has been determined that p65 phosphorylation at Ser536 assists in controlling the kinetics of NF-κB nuclear import during activation (Mattioli et al. 2004; Moreno et al. 2010). As a result of this difference in the kinetics of these two phosphorylation events, it is possible that the increased Ser536 phosphorylation that is seen following FASN knockdown is a consequence of an initial increase in NF-κB activation that is seen with FASN knockdown, but that it is in fact the presence of Thr254 phosphorylation and activity of Pin1 in the nucleus downstream of initial NF-κB activation that predominates in regulating p65 protein stability. If this is indeed the case, it would suggest that IKK mediated phosphorylation of p65 at Ser536 is not essential for maintaining p65 protein stability, and that constitutive nuclear accumulation of p65 could be maintained in the absence of this phosphorylation event.

4.10. Caveats and Limitations

There are caveats and limitations to the experimental methods utilized in this thesis. First, with regard to the cell lines utilized throughout the study pursued in this dissertation, there is somewhat a lack of consistency. For example, though MDA-MB-468 and MDA-MB-231 cells were utilized as supporting parental breast cancer cell lines for experimental data obtained for M3K cells, MDA-MB-436 cells with stable FASN overexpression were used as supporting cells for data obtained for M3K cells with stable FASN knockdown. Attempts were made to overexpress FASN in the MDA-MB-231 cells; however, these attempts were ultimately unsuccessful. Subsequently, as a result of the inability to overexpress FASN in the MDA-MB-231 cells, the MDA-MB-436 cells were chosen for stable FASN overexpression as a result of their low level of FASN expression and representative nature similar to MDA-MB-231 cells of the clinically relevant TNBCs. Regardless, it is worth acknowledging that a more appropriate choice for stable FASN overexpression would have been to use the MDA-MB-231 cells for a more direct comparison between studies using parental cell lines and studies using genetically-manipulated cells.

In addition, the results presented could have benefited from supporting studies using a second cell line with stable FASN knockdown. As the M3K doxorubicin resistant cells likely have many altered signaling pathways resulting from their stepwise drug resistance selection, it would likely be prudent for parallel experiments to be performed using the M3K/shFASN cells along with a second knockdown line, such as MDA-MB-231 cells with stable FASN shRNA

knockdown. This would provide the benefit of confirmation that the many altered signaling pathways in the M3K cells are not affecting results obtained in experiments with FASN knockdown. However, it is worth noting that previous attempts in our lab to produce stable FASN knockdown lines in parental cell lines have proved unsuccessful due to the reliance of the cancer cells on FASN for survival. As a result, it is possible that any studies utilizing FASN knockdown may require transient transfection.

In **Figure 8**, the experimental design involving the use of exogenous palmitate to affect total p65 levels has limitations. Specifically, in order to verify the role of palmitate in affecting total p65 level, a supporting control experiment will ultimately be necessary, wherein a free fatty acid quantification assay will need to be performed following the treatment of the M3K/shFASN and M3K/shScr cells with increasing concentrations of exogenous palmitate for 48 hours in an effort to verify that the treatment vehicle for palmitate and treatment duration is sufficient to allow for palmitate to enter cells.

With additional regard to **Figure 8**, while a range of palmitate concentrations was initially used in this experiment, and it was determined that noticeable cell death did not occur until concentrations approached 100 μM, experimental evidence to verify the lack of an effect on cell viability at the experimental concentration (30 μM) was not provided in this thesis. As a result, this figure would benefit from the addition of a supporting experiment, wherein a cell viability assay could be performed using increasing concentrations of

exogenous palmitate to determine concentrations at which cell viability is compromised in the M3K/shFASN and M3K/shScr cells.

In some areas, experimental design in this thesis project could have also benefited from the use of additional controls. One such area that has application to multiple experiments within this thesis involves experiments wherein drug treatment or genetic manipulation was used to determine subsequent effects on total p65 following stable FASN shRNA knockdown. Examples of this type of experiment include **Figure 22** and **Figure 23**, among others. In these experiments, an additional control could have been utilized, wherein the implicated genetic manipulation or drug treatment was also applied to the M3K/shScr cells in addition to the M3K/shFASN cells. This control would be useful in that it would allow for the determination of any potential direct effects of the genetic manipulation or drug treatment on total p65 as opposed to an effect that is seen in direct relation to the effects on total p65 that are mediated by FASN.

In **Figures 18-21**, TNF-α stimulation was utilized in an effort to induce detectable levels of phosphorylation of p65 at Ser536, and TNF-α was chosen to stimulate NF-κB in this context due to its previously characterized relationship with FASN. However, it must be noted that TNF-α is a promiscuous cytokine that can activate a variety of different signaling pathways involved in regulating both pro-survival and pro-apoptotic mechanisms (Wang and Lin 2008). As a result, utilizing TNF-α in these experiments creates a caveat, in that a variety of cell signaling pathways may be activated in response to TNF-α treatment, and these

signaling pathways may be affecting the relationship between FASN and p65. To attempt to alleviate potential concerns associated with TNF-α treatment, an additional control could be utilized in future experiments, wherein mirrored experiments are performed without TNF-α to provide evidence that TNF-α is simply magnifying effects on NF-κB downstream of FASN rather than inducing effects independent of and unrelated to the FASN-mediated mechanism.

4.11. Future Directions

The data and conclusions presented in this thesis have expanded upon a previously developed hypothesis and cell signaling mechanism regarding the role played by the oncogenic enzyme FASN in breast cancer cells. Though a previously unknown mechanistic link between FASN and NF-κB/p65 in breast cancer cells was uncovered, wherein FASN negatively regulates p65 protein stability, further research and exploration is required to fully understand the scope of this signaling pathway involving FASN and NF-κB/p65.

As described in section **4.8**, one of the most unclear areas of this mechanism involves the cellular consequences of the negative regulation of NF-κB/p65 exerted by FASN. As a result, it is necessary to uncover what role NF-κB signaling plays in breast cancer cells in the context of this FASN-mediated mechanism. A logical place to begin with these investigations would be to utilize the functional endpoints of cell death and DNA repair that Dr. Zhang's lab has previously explored with respect to this mechanism. To this end, overexpression of p65, as well as gene silencing or inhibition of NF-κB signaling could be used in

conjunction with FASN genetic manipulation and FASN inhibition to determine how manipulation of NF-κB signaling affects the ability of FASN to impact breast cancer cell death and NHEJ DNA repair activity. I hypothesize that NF-κB/p65 signaling will negatively affect both breast cancer cell viability and NHEJ DNA repair activity due, in part, to the previously established effects of NF-κB/p65 in negatively regulating PARP1 expression and activity, which has been found to mediate the effects FASN exerts on DNA repair and cell survival (Wu et al. 2016). If future research determines that NF-κB/p65 activation in the FASN signaling mechanism is acting in a pro-apoptotic manner, subsequent studies could investigate a potential ability for NF-κB/p65 to promote cell death mechanisms through Fas ligand signaling based on the characterized link between NF-κB and Fas ligand following DNA-damaging drug treatment (Kasibhatla et al. 1998). These experiments could also further be expanded to include treatment of cells with DNA-damaging drugs, including doxorubicin as an additional variable to specifically characterize the relationship between FASN, NF-κB, and resistance to DNA-damaging agents.

Another future aim within this mechanism that requires further exploration is the relationship between FASN, phosphorylation of p65 at Thr254, and Pin1. I was able to show in this thesis that Thr254 is important in FASN regulation of p65 protein stability, wherein it appears likely that FASN is able to suppress Thr254 phosphorylation of p65. I was also able to link FASN and p65 protein level to the expression and activity of Pin1. However, further research is required to establish a direct link between FASN, p65, and Pin1. To this end, future

research could expand the cycloheximide chase assay that was utilized in **Figure 21**. Initially, a parallel experiment could be performed using the MDA-MB-468 and MDA-MB-231 cells, wherein these cells would be transfected with either WT-FLAG-p65 or FLAG-p65-T254A followed by 0-12 hour cycloheximide chase to confirm that mutation of Thr254 of p65 disrupts p65 protein stability in parental TNBC cell lines in addition to the observed effects in M3K cells with stable FASN shRNA knockdown. Subsequently, an experiment could be pursued, wherein the MDA-MB-468 and MDA-MB-231 cells would be transfected with WT-FLAG-p65 in the presence or absence of siRNA targeting Pin1 or pharmacological inhibition of Pin1 prior to cycloheximide chase to determine the impact of the loss of Pin1 expression and activity on p65 protein stability in breast cancer cells as a means of linking Pin1 to p65 protein stability. Experiments could also be performed to link Pin1 and Thr254 phosphorylation of p65, in that co-immunoprecipitation assays involving Pin1 and p65 could be pursued in the presence or absence of WT-FLAG-p65 or FLAG-p65-T254A transfection to determine if mutation of p65 at Thr254 disrupts the interaction of p65 and Pin1.

Additionally, a clear caveat to the results summarized in this thesis is my inability to specifically determine that changes in FASN expression level can impact the level of p65 Thr254 phosphorylation. Multiple experimental methods were utilized in an effort to investigate this hypothesized causal relationship, including stimulating M3K/shFASN cells, along with their controls cells, as well as MDA-MB-436/FASN cells, along with their control cells, with TNF-α and performing Western blot analysis, wherein blots were probed with a p65 Thr254-

specific antibody. Unfortunately, though several commercially available antibodies were tested, none effectively recognized this phosphorylation site so as to definitively measure the phosphorylation level at this site (data not shown). Further, I also attempted to utilize an antibody specific to phosphorylated threonine residues that are followed immediately by a proline residue (Cell Signaling). Using isogenic cell lines with FASN expression changes as described above, I performed immunoprecipitation to specifically pull down and isolate p65 proteins from total protein lysate and subsequently performed Western blot for p-Thr-Pro. However, while I was able to confirm that p65 was able to be pulled down and isolated from total protein lysate, the p-Thr-Pro antibody failed to specifically recognize the immunoprecipitated p65 (data not shown). Though these methods were unsuccessful in attempting to determine the relationship between FASN expression changes and Thr254 phosphorylation of p65, future research could utilize a mass spectrometry approach, wherein stable isotope labeling can be used within multiple cell pools (such as with an shRNA knockdown cell line and its scrambled shRNA control cell line) to subsequently be able to directly compare the intensity ratios of the MS peaks that are obtained for each cell pool as a means of direct quantitation of the phosphorylation level of p65 at the Thr254 site (Oda et al. 1999).

If utilization of this technique was able to determine a direct causal relationship between FASN and the negative regulation of Thr254 phosphorylation level of p65 protein, an interesting research path would involve investigation into the molecular players involved in mediating changes in p65

Thr254 phosphorylation downstream of FASN expression. As discussed in section **4.3**, PKC is a kinase that has been linked to both FASN and NF-κB/p65. As a result, exploration of this mechanism would benefit from the investigation into the potential relationship between FASN, PKC, and p65 Thr254 phosphorylation. A simple way to investigate this would be to utilize gene silencing for specific PKC isoforms or pan-kinase inhibition for multiple PKC isoforms and measure p65 Thr254 phosphorylation levels in breast cancer cells in response to these manipulations. If pan-PKC inhibition did appear to affect p65 Thr254 phosphorylation, as well as total p65 protein levels, the activity of specific PKC isoforms towards p65 could subsequently be investigated using an *in vitro* kinase assay.

Further, if PKC or another kinase was able to be implicated in the signaling mechanism between FASN and NF-κB/p65, it would subsequently be important to investigate the role of palmitate, downstream of FASN, in regulating the kinase responsible for regulation of p65 expression. To initially investigate the role of palmitate, an experiment similar to that shown in **Figure 8** of this thesis could be employed, wherein exogenous palmitate treatment could be utilized to determine the effects on kinase activation and activity. If palmitate was able to be implicated using such an experiment, the acyl-biotin exchange assay could be utilized to determine if the responsible kinase is palmitoylated as a means for the effects exerted by FASN on that kinase.

Future research could also be devoted to further delineating the mechanism responsible for driving the degradation of p65 in the presence of high

FASN expression in breast cancer cells. While the data presented in this thesis shows that FASN induces p65 degradation through the proteasome following poly-ubiquitination of p65, the specific molecular players in this process remain unknown. As a result, it would be interesting to investigate which players in the ubiquitination and degradation cascade are involved in the process of FASN-mediated p65 degradation.

Interestingly, while the first E3 ubiquitin ligase found to be responsible for mediating poly-ubiquitination of p65 was SOCS-1, many E3 ubiquitin ligases have been determined to mediate p65 poly-ubiquitination and subsequently play a role in driving p65 proteasomal degradation, including PDLIM2, PPARγ, inhibitor of growth 4 (ING4), and human herpesvirus 8 (ORF73) (Ryo et al. 2003; Tanaka et al. 2007; Hou et al. 2012; Hou et al. 2014; Rodrigues et al. 2009). As a result, it would be interesting to investigate whether there is a specific E3 ubiquitin ligase that conjugates ubiquitin to p65 in the context of FASN regulation of p65 in breast cancer cells. If this is the case, it would provide useful and significant insight into the level of context dependence of the activity of E3 ubiquitin ligases and ubiquitination as a whole with respect to p65. This potential context dependence could be investigated quite simply by performing co-immunoprecipitation experiments using p65 and the various E3 ubiquitin ligases known to have a relationship with p65, as well as by utilizing gene silencing of the E3 ubiquitin ligases to determine the effects on p65 protein expression, stability, and NF-κB activity in the presence of either knockdown or overexpression of FASN.

4.12. Conclusions and Significance of Findings

The findings presented in this thesis expand upon the mechanistic role of the oncogenic enzyme FASN in breast cancer, allowing for a previously undetermined role for FASN in suppressing NF-κB/p65 expression through a reduction in its protein stability via proteasomal degradation to be uncovered. Although FASN-targeted small molecule inhibitors have seen little clinical success to this point, FASN remains a well-studied target for the development of anti-cancer drugs and a key marker of tumor prognosis and clinical outcomes. As a result, the determination of the mechanism behind FASN activity in cancer cells presented here will be important for the field of breast cancer research, in that further delineating the key molecular players involved in the ability of FASN to regulate tumor growth, as well as resistance to anti-cancer agents, will allow for the potential determination of other targets that may be druggable both in concert with FASN-targeted drugs, as well as in conjunction with various chemotherapeutic agents. Exploring these avenues could allow for more clinical success in targeting the FASN pathway than has been achieved in the past with small molecule inhibitors of FASN.

APPENDIX

Appendix A. The relationship between FASN, protein palmitoylation, and NF-κB/p65 is unclear.

In this thesis, it has been shown that FASN negatively regulates NF-κB/p65 by disrupting its protein stability. Further, the effects on NF-κB/p65 expression were shown to be reversed by reducing FASN expression, as well as inhibiting FASN activity. This mechanism also appears to specifically involve the phosphorylation site Thr254 of p65, as well as a p65 binding partner, Pin1. However, the upstream mechanism directly responsible for the ability of FASN to mediate these effects on p65 and p65 phosphorylation remains unclear.

As described earlier, FASN can impact cell signaling events through protein modification, specifically through a process known as protein palmitoylation, wherein a 16-carbon palmitate moiety is added to a cysteine residue. It has been shown in this thesis that exogenous treatment of breast cancer cells with the product of FASN catalysis, palmitate, results in a decrease in p65 protein level, indicating a specific role for palmitate in the mechanism. As a result, I hypothesized that protein palmitoylation may play a role in regulating the effects of FASN on p65, in that high levels of FASN expression may result in increased palmitoylation of certain proteins or p65 itself, which could then prevent p65 Thr254 phosphorylation and lead to disrupted p65 protein stability.

In order to investigate the potential relationship between protein palmitoylation and p65 in breast cancer cells, the palmitoylation inhibitor 2-bromopalmitate (2-BP), which acts as a palmitate analog to block global protein

palmitoylation upon treatment, was utilized (Webb et al. 1999). As shown in **Figure A-25**, when M3K doxorubicin-resistant breast cancer cells were treated with 6 μM 2-BP for a period of 48 hours, total p65 protein level decreased compared to vehicle-treated cells. This result likely indicates that direct palmitoylation of p65 is not responsible for the effects of FASN on p65 protein stability, as, if this were indeed the case, high FASN level would be hypothesized to induce increased p65 palmitoylation to reduce p65 protein stability, which would suggest that inhibition of palmitoylation would result in an increase in total p65 level. However, this result does raise additional questions that are explored in section **4.3**.

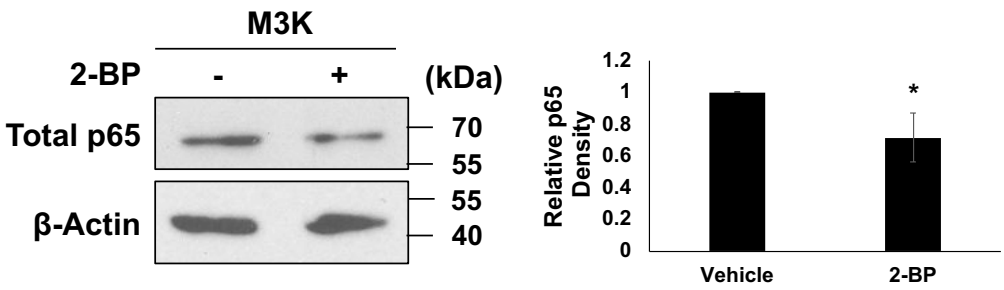


Figure A-25. The relationship between FASN, protein palmitoylation, and NF-κB/p65 is unclear.

Western blot analysis of M3K drug-resistant breast cancer cells treated with 2-BP at 6 μM or vehicle (DMSO) for 48 hours. Membranes were probed for total p65 for effects of 2-BP. Images were quantified using ImageJ. *$p<0.05$. Graph is representative of three independent experiments. Error bars represent standard deviation from the mean.

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CURRICULUM VITAE

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EDUCATION

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PROFESSIONAL EXPERIENCE

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AWARDS/FELLOWSHIPS

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PRESENTATIONS

Barlow, LJ (May 2018). Mechanism of FASN regulation of NF-κB/p65 expression in drug resistance of breast cancers. Poster presentation at the 2018 *Great Lakes Drug Metabolism and Disposition Group Meeting*.

Barlow, LJ (April 2017). Fatty acid synthase-mediated palmitate production impacts epidermal growth factor receptor signaling to regulate specificity protein 1 in breast cancer cells. Poster presentation at the 2017 *American Association for Cancer Research Annual Meeting.*

PUBLICATIONS

Barlow, L.J., Zhang, J. T. (2019). FASN negatively regulates NF-κB/p65 expression in breast cancer cells by disrupting its stability. (in preparation).

Wu, X., Dong, Z., Wang, C. J., **Barlow, L. J.,** Fako, V., Serrano, M. A., . . . Zhang, J. T. (2016). FASN regulates cellular response to genotoxic treatments by increasing PARP-1 expression and DNA repair activity via NF-kappaB and SP1. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.1609934113.